# FACTORS AFFECTING GREENHOUSE GAS PRODUCTION AND ENRICHMENT OF NOVEL MICROORGANISMS FROM S1 BOG, MINNESOTA

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By

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**FACTORS AFFECTING GREENHOUSE GAS PRODUCTION AND ENRICHMENT OF NOVEL MICROORGANISMS FROM S1 BOG, MINNESOTA**

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Go Yellow Jackets!

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# Chapter1
# Introduction

## 1.1 Background Information

Peatlands are a type of freshwater wetland that cover approximately 3 % of the land surface area on Earth (Gorham, 1991; Kolton, Marks, Wilson, Chanton, & Kostka, 2019). Peatlands are widely distributed around the world, especially in boreal or northern regions. Although peatlands cover a relatively small global land area, they comprise a significant carbon sink, contributing approximately 30% of terrestrial soil carbon storage (450 Gt)(Myers, Webster, Mclaughlin, & Basiliko, 2012). Peatlands are generally water-saturated and therefore become anoxic close to the soil surface (Limpens et al., 2008). It has been hypothesized that these freshwater wetlands will not only warm up but also dry out with climate change, thereby stimulating microbial respiration and the release greenhouse gases. Because of these characteristics, peatlands have drawn much attention from researchers for their potential to release greenhouse gases, carbon dioxide and methane.

Greenhouse gases warm the planet and their release has the potential to accelerate climate change. Carbon dioxide ($CO_2$) is the greenhouse gas that is of greatest concern to the public. Atmospheric $CO_2$ was found to increase from 278 ppm in 1750 to 410 ppm in recent years(Dlugokencky & Tans, 2016). Although methane, ($CH_4$), is found at much lower concentrations in the atmosphere than $CO_2$, it contains 45 times more warming potential over a 100 year time scale (Bridgham, Cadillo-Quiroz, Keller, & Zhuang, 2013; Forster et al., 2007; Neubauer & Megonigal, 2015). According to recent reports, global atmospheric methane concentration in 1866 was 722 parts per billion (ppb) and it rose to 1866 ppb in 2019 (NOAA May 5 2019). Further, the rate of $CH_4$ methane accumulation in the atmosphere is much higher than $CO_2$. Therefore, more attention should be paid to the controls of methane production and release.

Climate change has warmed the Earth's surface by approximately one-degree

Celsius since 1940 (NASA 2020). There is a red line in global warming. Scientists have predicted that human society is able to tolerate a temperature increase within 1.5 degrees Celsius. Once this threshold is exceeded, our health condition and normal body functions are in danger (McGushin, Tcholakov, & Hajat, 2018). Therefore, it is important to uncover more information about the effects that peatlands have on global warming.

### 1.2 Purpose of the study

This thesis research focuses on a large scale climate manipulation conducted in northern Minnesota. The 10 year whole ecosystem warming experiment, known Spruce and Peatland Responses Under Changing Environments (SPRUCE) aims to predict and model greenhouse gas emissions and the alteration of biological communities from peatlands under realistic climate change scenarios(Hanson et al., 2017). The goal of this thesis is not only to study potential feedbacks of peatlands to global change, but also to investigate how peatland microbial communities impact the such processes. Therefore, this thesis project was designed to investigate the environmental conditions that control the functioning of peatland microbiomes. The investigation required understanding of biogeochemical cycles and microbial ecology. Potential environmental parameters linked to microbial respiration and greenhouse gas emission include temperature, oxygen availability, redox potential, pH, organic substrates provision is applied in lab work to predict their potential effects on microbial metabolism. Overall, the purpose of my thesis research is to provide a predictive understanding of peatland microorganisms and their impacts on climate change, through greenhouse gas emission. If possible, I seek to propose some ideas to slow down the environmental changes occurring in peatlands.

# Chapter 2: Literature Review

## 2.1 Review of Peatland Biogeochemical Cycles and Impacts to Climate Change

Since peatland soils are largely saturated with water and rich in organic matter, oxygen is depleted close to the surface and anaerobic conditions prevail below ground (Warren, Hergoualc'h, Kauffman, Murdiyarso, & Kolka, 2017). Therefore, biogeochemical cycles in peatland soils, and in particular the carbon cycle, are mediated by anaerobic microbial processes. In many other anaerobic environments, such as in the deep ocean or forest soils, inorganic electron acceptors may facilitate the carbon cycle and the degradation of organic matter through the respiration of sulfate, nitrate, and metals (Slobodkina et al., 2017; Wind & Conrad, 1995). However, inorganic electron acceptors are present in low concentrations belowground in peatland soils (Ralf Conrad, 2009). Therefore, the consensus is that methanogenesis serves as the main terminal electron accepting pathway during organic matter decomposition in peatlands (Bridgham et al., 2013). Methanogenesis is catalyzed by microbes belonging to archaeal domain. As introduced above, since methane has strong warming potential and considering that peatlands represent a substantial source of methane to the atmosphere, it is important to understand the belowground carbon cycle and the controls of methane emission in the peatlands. In sphagnum spp. dominated peatlands, lignin-like phenolics and lignin derived compounds are always found because they are close related to the function and structure of sphagnum(Bengtsson, Rydin, & Hájek, 2018). Detailed processes will be reviewed later.(Drake, Horn, & Wüst, 2009)

## 2.2 Review of Organic Matter Degradation under Anoxic Conditions

The degradation of organic matter in soils under anoxic conditions is driven by a

complexity of anaerobic microbial processes (Zehnder & Mitchell, 1978). During the process, complex carbon polymers are first hydrolyzed into monomers such as glucose. Monomers then go through fermentation to produce short chain fatty acids alcohols, hydrogen, and carbon dioxide. These compounds are considered as intermediates in anaerobic degradation (Figure 1).

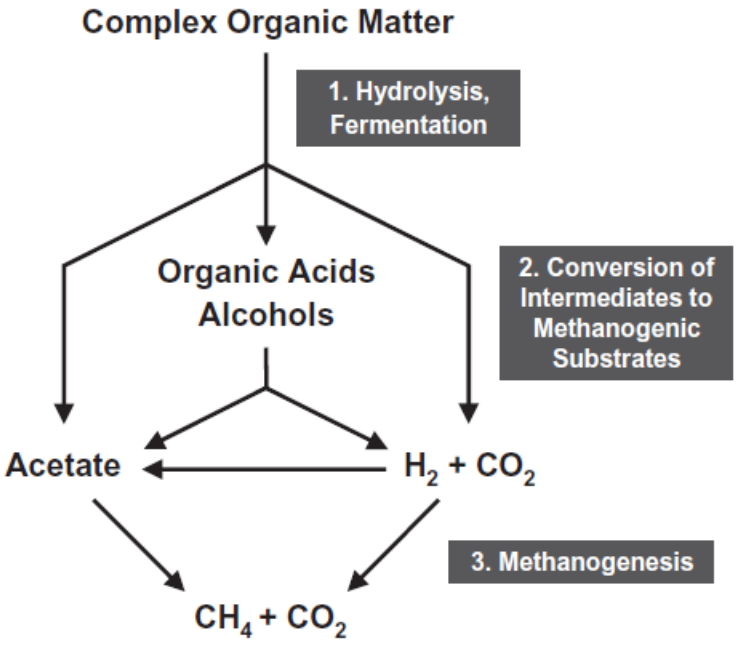



Figure1 : Simplified conceptual model of anaerobic organic matter degradation whereby methane and carbon dioxide are produced from complex organic matter ((Zehnder & Mitchell, 1978) (Drake et al., 2009)(adapted)).

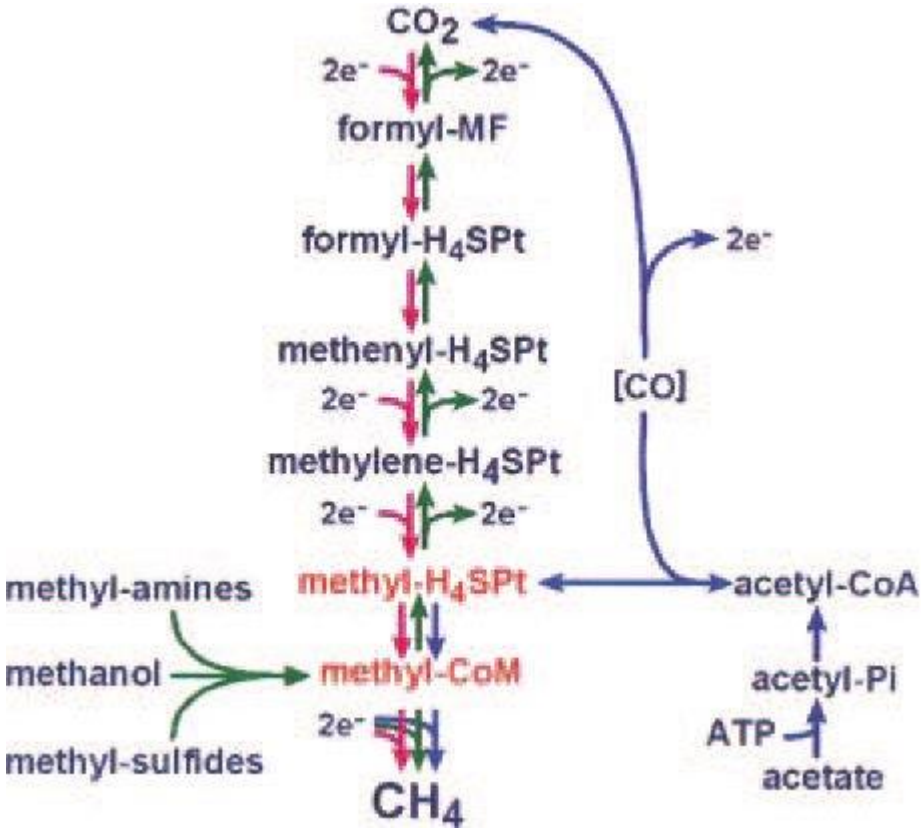


Figure 2: Metabolic pathways of methanogenesis.(Galagan et al., 2002)

Finally, the products of fermentation are terminally metabolized to $CO_2$ and $CH_4$ through respiration reactions. Oxygen supplies the largest amount of free energy and therefore it is respired first by microbes (James, 1946). In the absence of oxygen, fermentation and respiration are the main pathways for microorganisms to gain energy. Generally speaking, fermentation does not require an external electron acceptor, and carbon substrates such as carbohydrates serve as the electron donor and electron acceptor simultaneously (Morimoto et al., 2004). Under aerobic conditions, respiration with oxygen is always favored by microorganisms since oxygen is the most efficient electron acceptor.

As mentioned above, fermentation as well respiration produces $CO_2$. Therefore, for the purpose of simplification, in the following text, $CO_2$ production by microorganisms will be collectively called anaerobic respiration, with the exception of places in the thesis where fermentation and respiration are distinguished based on

experimental data and results.

In peat samples from southern peatlands in North Carolina, addition of glucose significantly increases the carbon dioxide production under anaerobic condition, indicating glucose can still stimulate the respiration process even under anaerobic conditions. When hydrogen or acetate is provided anaerobically, the production of carbon dioxide was not inhibited while $CO_2$ production pathways were totally blocked under aerobic condition. Also, while pH was increased by 2 comparing with the ambient pH in the peatlands, as twice $CO_2$ production was observed as natural condition(Bridgham & Richardson, 1992). As previously reviewed, our study site has great amounts of lignin-like compounds. Recent research revealed that under certain conditions, when appropriate electron acceptors are provided such as sulfate, lignin could be degraded anaerobically to produce $CO_2$, although the process is pretty slow because lignin is kind of resistant to anaerobic degradation for its complex structure(Colberg & Young, 1985). Then the question turns to be, if no oxygen is included in the respiration process, which chemicals work as electron acceptors? Previous research revealed that humic substances, which are generally abundant in soil environments, can serve as electron acceptors for the respiration to complete(Keller & Takagi, 2013). Furthermore, quinone or quinone-derived compounds are also found to be a possible electron accepting agents, so that high carbon dioxide production is sustained(Lovley, Coates, Blunt-Harris, Phillips, & Woodward, 1996; Ye, Keller, Jin, Bohannan, & Bridgham, 2016).

### 2.3 Review of $CO_2$:$CH_4$ Ratio in Different Sites

Due to the fact that carbon dioxide and methane have different heat storage capacities in the atmosphere and are regulated by different biogeochemical controls, the ratio of carbon dioxide to methane is an important parameter to demonstrate the response of microbial metabolism to climate change in certain peatland environments. In the absence of alternate terminal electron accepting processes (TEAPs), the final $CO_2$ to $CH_4$ ratio of produced gases from reaction stochiometry

would be predicted to be 1:1. However, as shown in Table 1, previous studies which have measured this ratio in peatlands, either by in situ experiments or lab incubations, rarely observed a 1:1 ratio. Rather, a larger amount of $CO_2$ is almost always produced and/or released, and this ratio is a significant parameter to determine the degree to which other electron sinks influence the carbon cycle in peatlands.

Table 1: The ratio of carbon dioxide to methane measured in the field or in the laboratory for several different peatland environments around the world.

| Place | $CO_2$:$CH_4$ Ratio | Reference |
|---|---|---|
| Subarctic Alaska Peatlandns | 4~150 | (Wickland, Striegl, Neff, & Sachs, 2006) |
| Finland subarctic peatlands | 3~53 | (Huttunen, Nykänen, Turunen, & Martikainen, 2003) |
| Sweden, Stordalen Mire | 1.3-5 | (Hodgkins et al., 2014) |
| North Sweden Peatlands | 1.2-5 | (McCalley et al., 2014) |
| Russia Subarctic Peatlands | 4~20 | (Heikkinen, Elsakov, & Martikainen, 2002) |
| Sweden raised bog | 1.2-6 | (Waddington & Roulet, 2000) |
| Minnesota Peatlands | 6-15 | (Glaser et al., 2016) |
| Canada Northern wetlands | 10-20 | (Valentine, Holland, & Schimel, 1994) |
| Lake Agassiz Peatlands, Minnesota (In situ) | 0.6-1.3 | (Romanowicz, Siegel, Chanton, & Glaser, 1995) |

A large range in the $CO_2$:$CH_4$ ratio is observed between peatland sites and over time, from approximately 1 to over 100. Theoretically, in peatlands where high ratio was observed such as Alaska and Finland subarctic peatlands, other electron accepting processes besides methanogenesis likely mediate the terminal decomposition of organic matter. In contrast, in areas where the ratio is low such as

in raised bogs of Sweden and the Lake Agassiz peatland of northern Minnesota, it can inferred that methanogenesis is the predominant TEAP under oligotrophic conditions and where alternate electron acceptors are absent.

### 2.4 **Review of the Biochemistry and Ecology of Methanogenesis**

In the terminal degradation of organic matter under anaerobic conditions, fermentation products are metabolized through a variety of respiration reactions, including methanogenesis. Methanogenesis provides among the lowest free energy changes of any terminal electron accepting process and therefore this process dominates when other electron acceptors are absent. The biochemical pathways are shown in Figure 2 (R Conrad, 1999; Galagan et al., 2002; Tarvin & Buswell, 1934). In general, methanogenesis is a complex biochemical process, involving many proteins and considerable genetic regulation. The most common molecular marker for methanogenesis is the gene encoding methyl coenzyme M reductase (mcrA). This gene is considered to be a reliable indicator of the presence of methanogens. This gene encodes a protein that is able to reduce methyl coenzyme M reductase, which is the final step to produce methane in methanogenesis.

Methane production is generally believed to be carried in three traditional pathways: (i) a hydrogenotrophic pathway in which hydrogen oxidation is coupled to $CO_2$ reduction, (ii) an acetolactic pathway in which acetate is cleaved to carbon dioxide and methane, and (iii) a methylotrophic pathway in which methylated or methoxylated compound are cleaved to directly produce methane. Methylotrophic methanogenesis is the least understood of these pathways and more research is required to understand its significance. Here I review the ecology of methanogens, especially in peatland soils from all over the world. The focus will be on hydrogenotrophic and acetoclastic methanogens, since comprehensive knowledge of methylotrophic methanogenesis is lacking. The chemical reactions formula were listed as equation (1)-(3)(Lyu & Whitman, 2019)

Hydrogenotrophic [$CO_2+H_2 \rightarrow CH_4+2H_2O \quad \Delta G°'=-131KJ/mol$]

Equation(1)

Acetoclastic [$CH_3COO\text{-}+H_2O \rightarrow CH_4+HCO^{3-}$ $\Delta G°'$=-31KJ/mol]

Equation (2)

Methylotrophic [e.g. $CH_3OH \rightarrow 3CH_4+CO_2+2H_2O\Delta G°'$=-319KJ/mol]

Equation (3)

Rice paddy soils represent freshwater wetlands in which methanogenesis has been studied in detail. In enrichment cultures from rice paddy soils, the genus *Methanosarcina* was found to the highest relative abundance of methanogens, up to 50%-80%. Members of the *Methanosarcina* genus have been demonstrated to carry out acetoclastic methanogenesis, indicating that this is the dominant metabolic pathway in the rice paddy soil environment (Kato et al., 2015). According to (Yuan, Huang, Rui, Qiu, & Conrad, 2019), in rice field soils, *Methanosarcinaceae* was the most dominant archeal family, so acetoclastic methanogenesis was probably the most activity in rice paddy soils. *Methanocellaceae* was the second dominant with a significant portion, so that hydrogenotrophic methanogenesis activity was also considered to be significantly active in the environment. In addition, a significant portion of hydrogenotrophic methanogens was also detected, specifically members of the genera *Methanocella* and *Methanoculleus*, which made up approximately 30% of the methanogen community. In other studies of rice paddy soils (Lueders & Friedrich, 2000) as well as in the rice rhizosphere (Chin, Lüders, Friedrich, Klose, & Conrad, 2004), the methanogen community was shown to be dominated by acetoclastic methanogens of the *Methanisarcinaceae* with hydrogenotrophs of *Methanocellaceae*, also occupying a significant portion of the community.

Contradictory results have been obtained for methanogen communities in peatlands. In the S1 bog, which is the study site of this thesis research, it was found that *Methanosarcinaceae* dominated the shallower depth (<40 cm), while *Methanocellaceae* dominated the deeper depth, from which could be inferred that acetoclastic methanogenesis was favored at surface area of S1 bog while hydrogenotrophic methanogenesis was more active in deep S1 bog(Lin et al., 2014).

In a less oligotrophic, higher pH fen in southeast Germany, the genus *Methanosarcina* was found to dominate among other methanogens in enrichment cultures, comprising up to 80% of the relative abundance of methanogens. *Methanoregula*, which carries out hydrogenotrophic methanogenesis, was found to be the second most abundant, comprising approximately 20% of the relative abundance. All other methanogens were present in much lower abundance (Schmidt, Horn, Kolb, & Drake, 2015). In contrast, in the soils of oligotrophic and more acidic bogs (Cadillo-Quiroz et al., 2006) and a minerotrophic fen (Cadillo-Quiroz, Yashiro, Yavitt, & Zinder, 2008), methanogen communities were dominated by members of the *Methanoregulaceae*, hydrogenotrophic methanogens, with relative abundances of 80% and 50 %, respectively. According to the literature, the dominant methanogenesis pathway shows high variation among different environments, and predictions remain difficult. Therefore, more research is essential to determine the controls of methanogenesis.

# Chapter 3: Material and Methods

## 3.1 Site Description and Sampling

### 3.1.1 Site description

This study investigated porewater and peat (soil) collected from the S1 Bog, a 8.1 ha ombrotrophic bog, located in the Marcell Experimental Forest, near Grand Rapids, Minnesota, United States (47 30.4760 N; 93 27.1620 W; 418 m above mean sea level). At the S1 bog, plant communities are dominated by peat mosses of the genus Sphagnum spp. (Zalman et al., 2018). As previously introduced, the S1 bog is oligotrophic and acidic, pH of which was measured to be around and mostly below 4. Also, the environment below surface is anaerobic (Oxygen concentration ~ 20ppb) (Lin et al., 2014; M. J. Warren et al., 2017). The S1 bog is the site of a whole ecosystem warming experiment funded by the U.S. Department of Energy and known as the Spruce and Peatland Responses Under Changing Environments project, which investigates the ecosystem impacts of warming and elevated carbon dioxide under realistic scenarios (Y. Huang et al., 2017; R. Wilson et al., 2016). The SPRUCE experimental design is shown in Figure 3. The research site has multiple enclosures, that simulate warming and elevated carbon dioxide in a regression-based design (Hanson et al., 2017). Experimental treatments include +2.25 °C, +4.5°C, +6.75 °C, +9°C and either ambient or twice atmospheric $CO_2$. The goals for these treatments are to determine and predict the biological community change as global warming continues. However, in this thesis research, all peat materials used were located outside of the enclosures without any artificial treatment. Our goal is to determine how would natural microbial communities in S1 bog nowadays respond to given treatments.

Two trips were conducted to Marcell Experimental Forest in Summer 2019, one trip in early July and another in early August. During both trips, porewater and peat were collected for microbial community characterization and laboratory experiments.

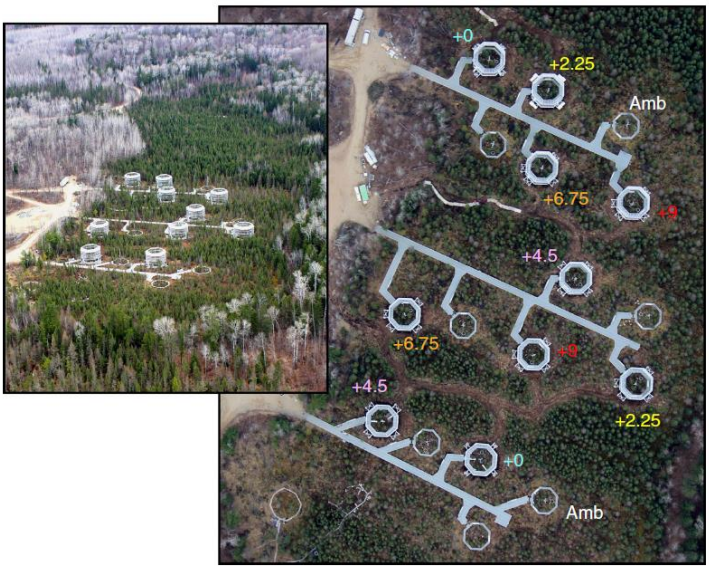



Figure 3: Aerial photographs showing the experimental design and distribution of experimental enclosures designed to mimic climate change drivers, temperature or elevated $CO_2$ concentration.(Adapted from Wilson et al. 2016)

### 3.1.2 Porewater Samples Collection for Gene Analysis

For microbial community analysis using molecular techniques, porewater samples for DNA and RNA extraction were collected from the following enclosures: ambient $CO_2$, +0 °C; ambient $CO_2$, + 4.5°C; ambient $CO_2$, +9°C; elevated $CO_2$, +0 °C; elevated $CO_2$, +4.5 °C; elevated $CO_2$, +9°C; ambient temperature and $CO_2$ without an enclosure. A portable peristaltic pump was used to collect the samples over a range of soil depths from pre-installed wells of PVC tubes. A day before collection, each well was pumped out to remove any contaminants and allow for recharge and to ensure fresh porewater. From each enclosure, the following volumes were collected into 50 ml Falcon tubes: 50 ml of porewater from 25 cm and 50 cm depth in one Falcon tube, 100 ml porewater from 100 cm depth in 2 Falcon tubes, and 200 ml from 200 cm depth in 4 Falcon tubes. There are two reasons for sampling different porewater volumes. First, less porewater is available from especially the middle depths due to a lower hydraulic conductivity. Second, it was shown

previously that microbial density declines with depth , and therefore more volume is required to obtain sufficient material for further analysis.

### 3.1.3 Processing of Porewater Samples for DNA and RNA Extraction

Samples collected were immediately stored at 4 degrees Celsius. Filtration and storage must start as soon as possible after collection, especially if the samples were targeted for RNA extraction. A Nalgene filtration tower connected to a vacuum pump was set up, and samples were filtered using a 0.22 um filter. An aliquot of 50-70 ml of porewater was filtered at a time and the total volume filtered was recorded. Samples collected from the same depth and site, even though collected in different Falcon tubes, were pooled into the same filter. Once filtration was complete, for DNA collection, filter paper was rolled and cut into several small pieces, placed into 2 ml Eppendorf tubes and immediately stored in the freezer. As for RNA collection, two methods were applied. No matter which protocol was followed, RNAlater® was prepared in advance. In one protocol, the RNAlater® and porewater sample were mixed in a 1:1 ratio were mixed prior to filtration and filtered together. In a second protocol, porewater was filtered first and then the filter was stored in a Eppendorf tube filled with RNAlater®. Immediately after filtration, samples were immersed in a cooler containing mixture of ethanol and dry ice, for flash freez of the samples. Once samples were frozen, they were transferred to another sealed cooler with dry ice and the whole cooler was stored in a freezer prior to transport back to the lab.

### 3.1.4 Porewater Collection for Cultivation Experiments

Porewater for the preparation of cultivation media was collected outside of the experimental SPRUCE enclosures at the site designated as “T3F” at approximately 25 cm depth (Lin et al., 2014). Thus, this site represents the ambient or natural condition, ambient temperature and ambient carbon dioxide content. Gas-tight tubings was employed with a portable peristaltic pump and a sipper. Porewater was pumped into 1-liter carbonate bottles. Once 4 bottles were filled, we moved to another spot approximately 2 meters away from the previous one to avoid

contamination and draining porewater from a single location. A total of 24 bottles of porewater were collected and stored in a cooler with blue ice for transport. Once they arrived in the lab at Georgia Tech, they were immediately stored in a cold room, where the temperature was set to 4 degrees Celsius.

### 3.1.5 Peat and Porewater Collection for Laboratory Investigations

During the second field trip in mid-August, 2019, the main task was to collect peat for incubations and enrichment cultures. Peat was collected with a Russian corer at T3F (Lin et al., 2014) at soil depths of 25 cm and 75 cm and stored in sterile Ziplock bags. Samples were flushed with nitrogen in an anaerobic bag to ensure anoxic conditions and placed in a cooler at approximately 4 °C. At the field lab of the Marcell Experimental Forest, samples were placed into an anaerobic chamber. A subsample was chosen from the center of the peat plug in the Ziplock bag and transferred to sterile 50 ml Falcon tubes to ensure aseptic conditions for cultivation. Falcon tubes containing full of fresh and clean peat with no headspace were tightly sealed in canning jars to strictly avoid gas change with outside environments and stored at 4 degrees Celsius for later use. The peat remaining in the Ziploc bag was sufficiently homogenized by hand, gas space evacuated, and stored at 4°C in a refrigeratoe. Porewater collected at T3F from depths of 25 cm and 75 cm was pumped directly into sterile 125 ml serum bottles that were immediately sealed with rubber stoppers and aluminum caps. Porewater samples were degassed with nitrogen for at least 20 minutes and stored in a cooler at approximately 4 °C. Porewater samples were shipped back on blue ice in a cooler to the lab and immediately stored at 4 degrees Celsius.

## 3.2 Greenhouse Gas Analysis by Gas Chromatography

A SRI 8610C gas chromatograph equipped with a HAYESEP® N column with a flame ionization detector and an in-line methanizer was used to track the concentration of gases, carbon dioxide and methane, in the headspace of cultures and incubations. The retention times of $CH_4$ and $CO_2$ were approximately 1.45 min and

2.63 min, respectively. A standard calibration was conducted each time before analysis. The concentration of gas standards used for standard calibration was 500 ppm for $CH_4$ and 1000 ppm for $CO_2$. Standard additions of 100, 80, 60, 40, and 20 ul of standard gas were injected into the GC separately, where 100 ul was considered to be the standard concentration ($CO_2$:1000 ppm, $CH_4$:500 ppm). Linear calibration curves were calculated using regression of concentration as ppm vs. peak area. In order to calculate the molar concentration of the gas from an unknown sample, the Ideal Gas Law (equation 4) was applied (Clapeyron, 1834):

$$PV = nRT \qquad \text{Equation(4)}$$

Where P is the pressure of the gas, V is the volume, n is the number of moles, R is the gas constant equal to 0.0821 L·atm/(mol·K), and T is the temperature in Kelvin.

Since $CO_2$ has a relatively high solubility in water, to more accurately state the overall $CO_2$ content in sealed incubations, Henry's law should also be applied (Equation 5). However, after applying Henry's law, it was found that correction for dissolved, aqueous $CO_2$ was extremely small in our case, which could almost be ignored:

$$[CO_2] = P/K_H \qquad \text{Equation (5)}$$

Where $[CO_2]$ is the dissolved concentration of $CO_2$, P is the partial pressure of $CO_2$, $K_H$ is the Henry's law constant, regarded as 29.41 atm/M.

Peak areas for $CO_2$ mostly ranged from 0 to 300 for concentrations from 0 to 1000 ppm. For methane, peak areas ranged from 0 to 160 for concentrations of 0 to 500 ppm. A volume of 100 ul of head gas sample was routinely injected into the GC. However, if the peak area was found to be significantly outside of the range of the standard, the injection volume was adjusted and the data normalized with a dilution factor afterward.

### 3.3 Analysis of Organic Acids by High Performance Ion Chromatography

A Dionex, DX-300 series ion chromatograph equipped with a Dionex IonPac® ICE-AS6 ion-exclusion was employed. The column is designed for efficient

separation of low molecular weight aliphatic organic acids based on an ion exclusion mechanism, which allows retention and separation of weakly ionized acids according to their different pKas (Szeinbaum et al., 2017). In this project, short chain fatty acids are of interest as intermediates in anaerobic degradation and for elucidation of biochemical pathways. After reviewing the literature, the following compounds were chosen for analysis: pyruvate, formate, fumarate, succinate, citrate, lactate, butyrate, acetate and propionate (J.-J. Huang, Chen, & Ruaan, 1991; Lamed & Zeikus, 1980; Schmidt et al., 2015). These compounds represent likely reactants and products of anaerobic degradation in soils. For example, glucose is believed to be fermented to succinate, propionate, butyrate and lactate (Drake et al., 2009; McInerney, Mackie, & Bryant, 1981). Standard chemicals were chosen from Sigma-Aldrich in sodium salt form and to meet chromatography standards. A 1mM standard was prepared with Type I reagent grade water with a specific resistance of 18.2 megohm-cm. Serial dilutions of a factor of 2 were prepared, with final standard additions of 500uM, 250uM, 125uM. For mixed standards, 200 ul of DI water was added into a 2 ml HPLC vial along with a 200 ul aliquot of stock solution for each short chain fatty acid mentioned above prepared to a concentration of 10 mM, resulting in 1 mM final concentration for each fatty acid. Then, similar to single standards, serial dilutions were conducted in duplicate to obtain standard additions of 500 uM, 250 uM and 125 uM mixed solutions. Standards were used immediately or stored at 4 C for later use. In the beginning, mixed standard samples were run on the IC and then each single short chain fatty acid standard was run separately to match the corresponding retention time for each analyte in single and mixed standards. Standard calibration curves for each short chain fatty acid were plotted by concentration versus peak area of conductivity (uS) with a MATLAB program.

For analysis of culture samples, one milliliter of liquid was removed from each serum bottle and transferred to 1.5 ml tube. If the sample contained peat, it was centrifuged at 9000 rpm for 20 minutes. If the sample did not contain peat, a centrifuge speed of 9000 rpm was applied for 5 minutes. After centrifuging,

supernatant was carefully transferred to another 1.5 ml tube and then filter sterilized with a 0.22 um filter into another new 1.5 ml tube in the anaerobic chamber. Samples were immediately frozen for future use or stored at 4 degrees for immediate use. Each time organic acid analysis was performed, a standard calibration curve was constructed with serially diluted mixed standards, and 100 ul of sample was injected. Organic acid constituents of each sample were determined by the retention time of each peak and the concentrations were determined by their peak area. Between runs, the sample syringe was washed with nanopore water three times and the loop of IC was rinsed once.

### 3.4 Enrichment of Anaerobic Microorganisms

A major goal of this thesis research was to enrich novel microorganisms from peat and porewater samples. Two types of microorganisms were targeted, methanogens and members of the *Acidobacteria*. The significance of methanogens is obvious in order to learn more about the anaerobic degradation process happening in the peatland. Very few methanogens have been isolated from peatlands and the enrichment of novel species will expand our understanding of the physiology of these organisms. *Acidobacteria* were targeted because this phylum of bacteria was shown to be dominant in peatland environments with molecular techniques (Kolton 2019, Lin 2014), but few species have been cultivated and their metabolism remains mostly unknown. An improved knowledge of the physiology of *Acidobacteria*, especially under anaerobic conditions, will undoubtedly shed light on the belowground carbon cycle in peatlands (Dedysh & Sinninghe Damsté, 2001)

Initially, since few anaerobes have been enriched from peat soils, an undefined medium with complex carbon was employed. The R2A medium was composed of: proteose peptone, 0.05%, casamino acids 0.05%, yeast extract, 0.05%, dextrose, 0.05%, soluble starch, 0.05%, dipotassium phosphate, 0.03%, magnesium sulfate, 0.005%, sodium pyruvate, 0.03% (w/v). The R2A medium used here was diluted 10 times in 300 ml porewater collected from S1 bog in July 2019 to serve as culture

medium. According to the instructions, full strength R2A consists of 3.12 g of R2A broth dissolved in 1000 ml solvent, and thus 0.312 g in 1000 ml solvent was considered as a 10 times dilution. The purpose for using R2A as initial enrichment medium was to enrich microorganisms with a broad range of substrate utilization. The pH of the medium was adjusted to 4, close to the in situ pH (Lin et al., 2014), with 1 M Hydrochloride (HCl) and filter sterilized with a disposable filter tower with Nalgene filter bottles. Filtered medium was transferred to 500 ml autoclaved glass bottles in a sterilization cabinet to reduce the possibility of contamination. The glass media bottle was then sealed tightly with a rubber stopper and lid, and flushed with nitrogen gas for 60 minutes. One day before setting up enrichment cultures, peat that would serve as the inoculum, porewater enrichment R2A medium, syringes, needles, scoops, rubber stoppers and serum vials were transferred into a Coy anaerobic chamber to incubate overnight in dark to ensure removal of trace oxygen. After flushing and placement into the anaerobic chamber after overnight, 0.5 M Homopipes was added as a buffer and 0.83 mM Titanium (III) nitriloacetic acid was added as a reducing agent. The prepared medium was used immediately or stored at 4 $^{o}$C for later use.

To initiate each enrichment culture, 0.5 g of peat stored in Falcon tubes was added into a 25 ml autoclaved serum vial in the anaerobic chamber and the vial was closed with rubber stopper. Ten milliliters of R2A medium was then added into each vial using a sterile syringe and the vial was sealed with an aluminum cap. Vials containing cultures were removed from the anaerobic chamber and flushed with nitrogen for another 5 minutes to assure positive pressure and then into an incubator set to 25 $^{o}$C.

For passaging, cultures were transferred to fresh media with a 10 % inoculum. In other words, 1 ml of mixed slurry was passaged every 30 to 45 days to assure viability, eliminate the solid phase peat constituents, and to increase the relative abundance of targeted microorganisms.

After several successful transfers in diluted R2A media, no solid phase material

could be observed in the cultures. A more specifically enrichment on our targeted microorganisms would be excercised.

### 3.5 Incubations with Amended Carbon Substrates

In this part of research, peat was the only candidate for inoculum because porewater inoculated culture was found to be not able to produce methane at all from previous experiences. To start up, porewater was filter sterilized with vacuum filter bottles and the filtrate was transferred to glass bottles in antibiotic fume hoods and sealed with rubber stoppers and lids immediately. The medium was then flushed for an hour. For each treatment, triplicates were applied. 0.5 g peat was added into each serum vial in the anaerobic chamber then sealed with rubber stoppers and aluminum caps, following by addition of 10ml of filtered porewater. In the end, substrates stock solution was added into each corresponding tube to achieve the final concentration to start the incubation.

### 3.6 pH Calibration

In order to establish and maintain close to in situ conditions, it was necessary to control and to measure pH. There was a concern that pH medium and chemistry may change over time in the cultures. The pH of a sample was measured in two ways. First, larger volumes of media, during preparation or after initiation of the experiments, were used to measure pH with a Thermofischer Scientific pH meter. This approach was used at termination of the experiments or if sufficient culture volume was available. For this approach, 2.5 ml of culture was removed with a sterile syringe and transferred to a 10 ml serum bottles in the anaerobic chamber and sealed with rubber stoppers. The probe of the pH meter was then placed into the bottle for determination (the bottle was opened for pH measurement). A second method was to use pH strips, which were considered less accurate but required much less culture volume. pH strips and samples to be measured were moved into the anaerobic chamber. Approximately 0.1 ml of culture volume was sampled by sterile syringe and dropped on the test area of the pH strip. About 30 seconds to 1 minute should be allowed for samples and the strips to react and have the color fixed. Finally, color

shown on pH strips was compared with standard colors corresponding to a specific pH range.

# Chapter 4: Results

## 4.1 Time Required for Porewater Filtration

In order to evaluate protocols for sampling microorganisms from porewater for community characterization, the amount of time needed for filtration was recorded. Less than 10 minutes was required to complete filtration of 100 ml porewater from 200 cm depth. A total of 30 to 50 minutes was required to complete filtration of 100 ml of porewater from 100 cm. As for 100 ml porewater from 50 cm depth, filtration lasted approximately 1 to 1.5 hours. Further, 5 to 7 hours was needed to filter 100 ml porewater collected from 25 cm depth. Filtration time was believed to be positively correlated with abundance of microbes in the sample.

## 4.2 Pre-incubation

Porewater was initially targeted as a simpler system for investigation of in situ microbial processes in peatlands. The goal for pre-incubation was to assess in situ rates of greenhouse gas production as well as to consume any substrates (e.g. electron acceptors and donors) residing in the porewater for downstream incubation experiments. Six porewater samples collected from 25 cm and 75 cm depth in August, which were planned to be used as inocula, were immediately flushed with nitrogen for 20 minutes after being transported back to the lab. After flushing, three porewater bottles from each depth were stored at 4 degrees and 14 degrees, respectively, resulting in 12 experimental cultures in total. The time zero started immediately after flushing. Methane and $CO_2$ concentrations were measured in the headspace of each serum vial by GC at days 0, 1, 3, 5, 8, 11, 14 during the first two weeks of incubation. Subsequently, gas measurements were performed once per week over 50 days total of incubation. During the incubation, no methane and little $CO_2$ was produced. After 50 days, 5 mM acetate was added to test for substrate limitation. However, one month after acetate amendment, no obvious stimulation in $CO_2$ production was observed

and methane was still not detectable in porewater samples incubated under any condition. At the end of the incubation, day 33 after acetate amendment, a small amount of methane production was detected in all three replicates collected from 25 cm depth and incubated at 14 ºC. Results from 25 cm porewater incubated at 14 ºC are shown in Figure 4. Results from the remaining treatments (75cm 14 ºC, 25cm 4 ºC, 75cm 4 ºC) are shown in Figure A5.

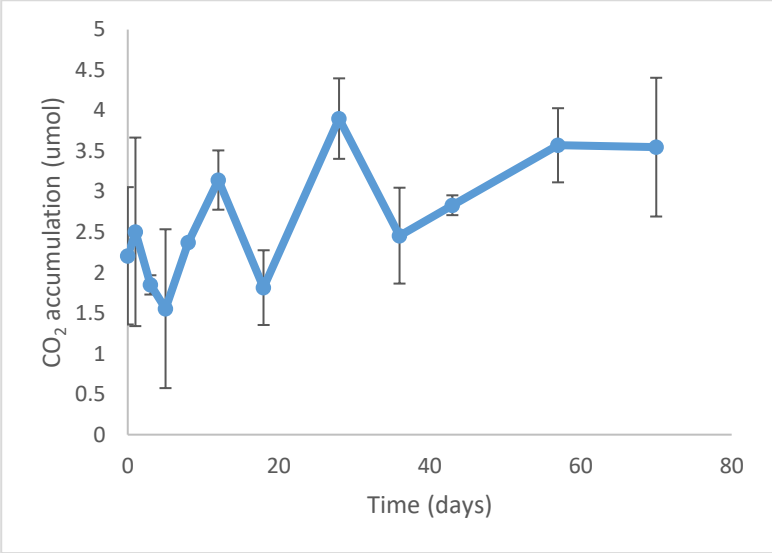



Figure 4: $CO_2$ production over time during pre-incubation of porewater collected from 25 cm at 14 ºC. At day 50, acetate was added to all treatments.

### 4.3 Reducing Agents

Microbial metabolism is likely regulated by temperature, pH, and reduction-oxidation chemistry in peatland soils. Redox potential, in particular, is a potentially important factor and yet it is difficult to maintain in anaerobic cultures or incubations. In S1 bog soils, where the environment is largely anoxic, it is essential to sustain a low redox potential in order to enrich specific microorganisms that are strictly anaerobic such as methanogens or Acidobacteria (Dedysh, 2011). Therefore, the question addressed here is which reductant would best stimulate the metabolic activities of anaerobic peat microorganisms. Four different reductants with the

following final concentration were chosen based on previous work: (1) 0.83 mM titanium nitriloacetic acid (Ti(III)Nta), (2) 0.83 mM Titanium citrate (Ti(III) citrate), (3) 1 mM sodium sulfide ($Na_2S$), and (4) 10 mM ferrous chloride. An 83 mM stock solution of Ti(III) citrate was prepared by mixing 4.8 ml of 0.5 M sodium nitrilotriacetate, 0.55 ml 15 % Ti (III) chloride solution dissolved in HCl (Sigma-Aldrich), and 7.2 ml of 0.4 M Tris HCl (pH=8) in an anaerobic chamber (Moench & Zeikus, 1983). In order to make an 83 mM Ti(III) citrate stock solution, 4.8 ml of 0.5 ml sodium citrate was used instead of sodium nitrilotriacetate and a 0.12 M sodium hydroxide solution (NaOH) was applied in place of Tris HCl. (Brauer 2006). The 100 mM $Na_2S$ stock solution was prepared in in degassed water in the anaerobic chamber and filter sterilized. It was recommended to cover the sulfide solution with aluminum foil to avoid light. Ferrous chloride salt was stored in the anaerobic chamber immediately after it was received to avoid oxidation. The stock solution was prepared by dissolving 5.08 g of $FeCl_2$ in 40 ml of degassed DI water and then it was autoclaved at 121 degrees Celsius for 20 minutes. After autoclaving, the solution was flushed with $N_2$ for 20 minutes and transferred back into anaerobic chamber to filter sterilize into a new bottle. All of the stock solutions above were filter sterilized and stored in a Coy anaerobic chamber before use.

A 10 % R2A medium was prepared as described above and used for the reducing agent experiment. For each treatment, a master mix was first prepared, where 0.312 g R2A medium was dissolved in 100 ml porewater and the pH was adjusted to 4. The medium was then filter sterilized using 250 ml filter bottles with a vacuum. After filtration, the medium was transferred to 250 ml glass bottles in the sterilization cabinet. The hood was UV sterilized for 20 minutes before use. Twenty-five milliliters glass vials were sealed with rubber stoppers and aluminum closures. Media were then flushed with nitrogen for an hour. All bottles and instruments used in the experiments were placed in the anaerobic chamber for at least overnight to minimize and eliminate oxygen residues. A group of autoclaved controls was prepared by sterilization at 121 degrees Celsius for 20 minutes after the medium was

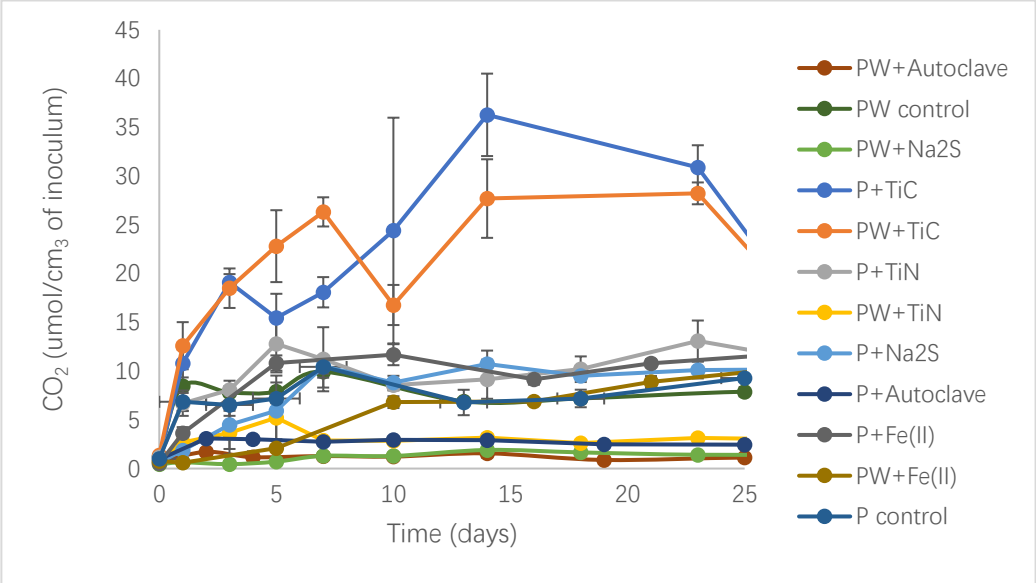
prepared and no reductant was added. Afterward, in an anaerobic chamber, 0.1% resazurin was added to each medium bottle along with 1 ml stock solution of each reductant to achieve a 100 times dilution. Once Ti(III)Nta, Ti Citrate or $Na_2S$ were added into the corresponding bottle, resazurin in the solution immediately turned clear. However, after $FeCl_2$ was added to each bottle, it took overnight for the resazurin to turn clear. Triplicates were prepared for each treatment. For each reductant, peat or porewater was applied as an inoculum separately. When peat was applied as an inoculum, 0.5 g peat was first added to each vial with an autoclaved spatula and the vial was sealed. Ten milliliters of the medium was then added. The cultures were briefly and gently shaken to homogenize. For cultures in which porewater only was used as the inoculum, serum vials were first sealed and then the inoculum was added using a sterile syringe. All cultures were then incubated at 25 degrees Celsius in the dark. Greenhouse gases were quantified in the headspace of each vial by GC analysis after 0, 1, 3, 5, 8, 11, and 14 days of incubation in the first two weeks then once or twice per week after. Other than gas production, $CO_2$:$CH_4$ ratios through time were also calculated. Results are shown in Figure 5. The gas accumulation results were normalized to umol of gas/$cm^3$ of inoculum (peat or porewater).

Enrichment cultures at day 6 and day 22 are shown in Figure 9. Microbial growth could be visualized as a biofilm on the bottom of the culture vial as well as turbidity in the medium, especially in porewater only cultures as no other solid phase was present. Biofilm in porewater inoculated cultures was first observed at day 3, in the cultures with Ti Citrate added as the reductant. At day 6, the biofilm was most pronounced in the porewater+Ti Citrate cultures and a biofilm was also detected in the porewater+TiNta cultures. As for cultures amended with sulfide and Fe(II), very little biofilm was observed. In the uninoculated controls, no biofilm was observed. At day 22, the following observations were made in reductant amended cultures: (i) Ti Citrate, biofilm was the largest among all treatments and appeared brownish (ii) TiNta, biofilm was less substantial. (iii) $Na_2S$, biofilm was pronounced

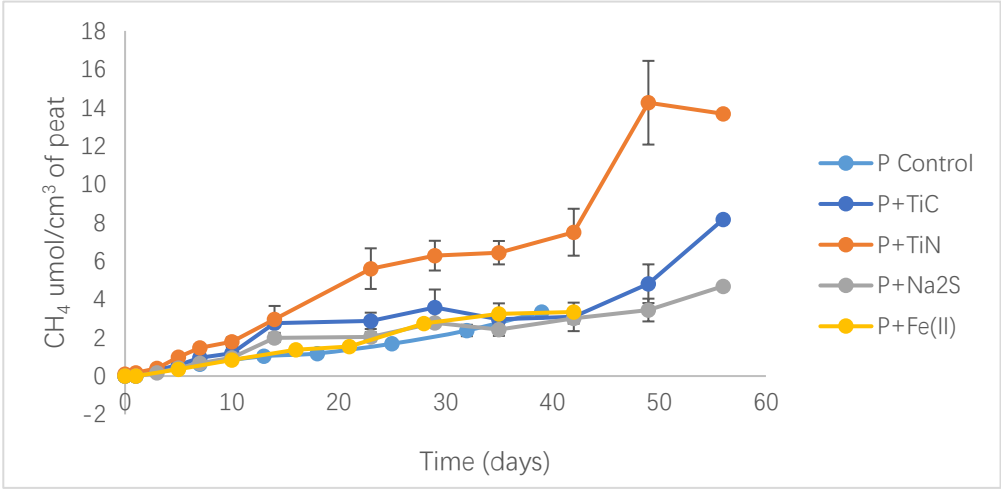
and appeared blue. (iv) $FeCl_2$, biofilm was small (v) Controls, biofilm was not observed at all.

pH was also measured during the cultivation period, but only for peat inoculated cultures. The initial pH was adjusted to 4 for all treatments. After 14 days, pH was the following: Peat+Ti Citrate cultures, 4.91; TiNta, 6.54; $Na_2S$, 5.40; $FeCl_2$, 3.89. At day 28, pH values were: Ti Citrate, 4.94; TiNta, 6.41; $Na_2S$, 5.35; $FeCl_2$, 3.81.

(a)




(b)



(c)

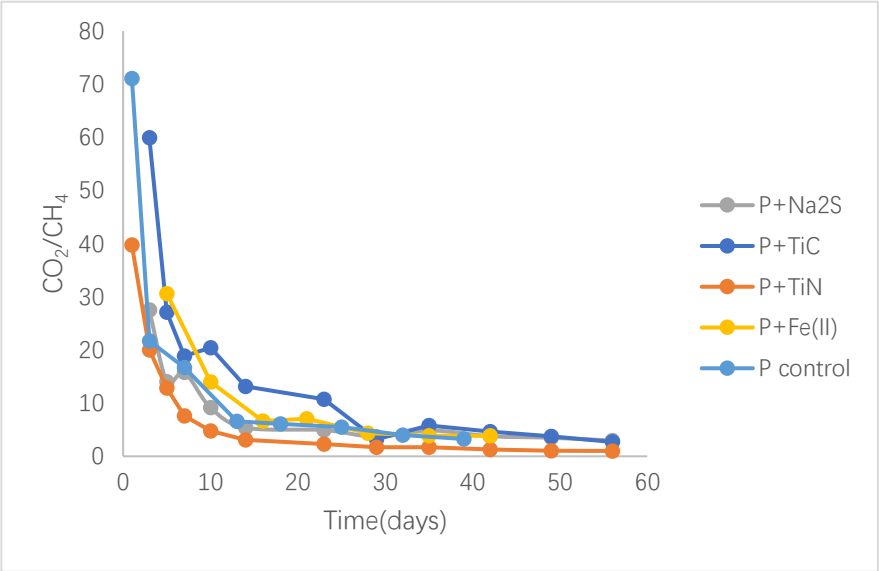


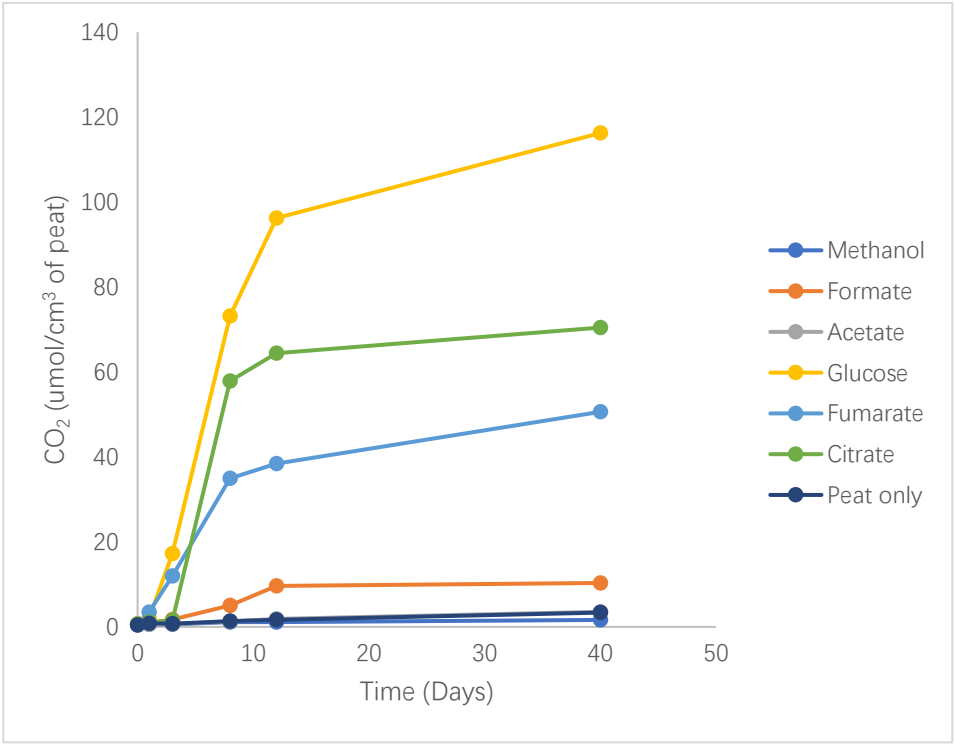

Figure 5: Greenhouse gas production in enrichment cultures amended with various reductants: (a) $CO_2$ accumulation with time with different treatments and inoculums (b) $CH_4$ accumulation with time for different treatments (c) Plot of $CO_2$:$CH_4$ ratio with time for different treatments. Values represent the average of triplicate cultures. Standard deviations are shown in (a) and (b). Control cultures were uninoculated. PW refers to porewater only cultures, whereas P refers to cultures inoculated with porewater and peat.

### 4.4 Substrate Utilization in Peat Microcosms

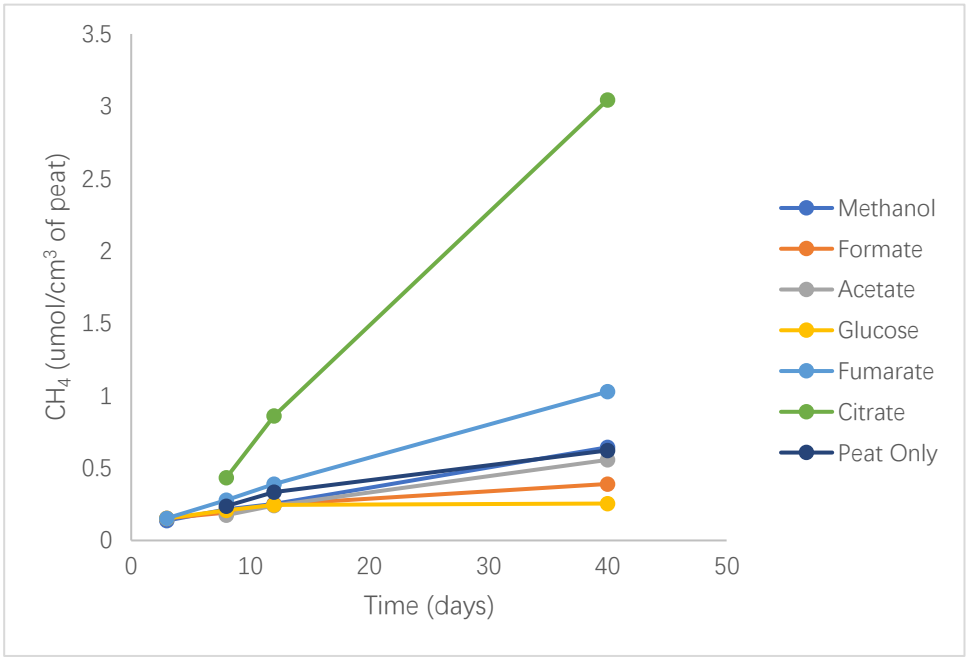
The objective of this part of the research was to determine which organic substrates, especially short chain fatty acids, are favored during the terminal metabolism for organic matter in S1 bog soils. Based on our previous work (Lin et al., 2014; 2015, Wilson et al., 2016; 2017; Zalman et al., 2017; Kolton et al., 2019), the following substrates were chosen: glucose, formate, fumarate, citrate, acetate and methanol. Glucose was chosen as a common carbohydrate monomer in the anaerobic degradation pathway that is not protonated at low pH. Formate was considered to be a hydrogen-like fermentation intermediate (Pinske & Sawers, 2016; Yoshida, Nishimura, Kawaguchi, Inui, & Yukawa, 2007). Acetate and methanol represent direct substrates for acetoclastic and methylotrophic methanogenesis pathways, respectively. Citrate was chosen after it was observed that it produced largest amount of $CO_2$ production of

any treatment in incubations amended with Ti Citrate. Fumarate was chosen as an potential alternate electron acceptor(Lin, Handley, Gilbert, & Kostka, 2015), but it could also possibly serve as fermentation substrate (Dunfield, Dumont, & Moore, 1993; Miller & Wolin, 1985; Ungerfeld, Kohn, Wallace, & Newbold, 2007). The forms of glucose and methanol were prepared from their solid and liquid forms, respectively. The remaining chemicals were prepared from their sodium salts (Sigma-Aldrich). Formate was initially prepared from the acid form but it was found to inhibit methanogenesis by GC analysis. Substrates were added to the following final concentrations: formate 10mM, acetate 5mM, fumarate 10mM, glucose 10mM, citrate 5mM, and methanol 10mM. For substrate utilization experiments, microcosms were prepared in serum vials to closely mimic in situ peats. In preliminary experiments, porewater alone produced little to no methane, peat was added as an inoculum to filtered porewater. Porewater was filter sterilized with a vacuum and the filtrate was transferred to glass bottles in a sterilization cabinet. Vials were immediately sealed with rubber stoppers and aluminum closures. The medium was then flushed with nitrogen for an hour. For each treatment, 0.5 g peat was added into each serum vial in triplicate in the anaerobic chamber and then sealed with rubber stoppers and aluminum caps, following by addition of 10 ml of medium. Lastly, substrates were added from stock solutions into each corresponding vial to achieve the final concentration to start the incubation. Microcosms were incubated at 25 degrees Celsius in dark. Greenhouse gases were quantified from the headspace of each vial by GC analysis at days 0, 1, 3, 5, 8, 11, 14 and once per week afterward. The results are shown in Figure 6.

(a)


140
120
100
80
60
40
20
0
0
10
20
30
40
50
CO2 (umol/cm3 of peat)
Time (Days)
Methanol
Formate
Acetate
Glucose
Fumarate
Citrate
Peat only

(b)


3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
CH4 (umol/cm3 of peat)
Time (days)
Methanol
Formate
Acetate
Glucose
Fumarate
Citrate
Peat Only

(c)

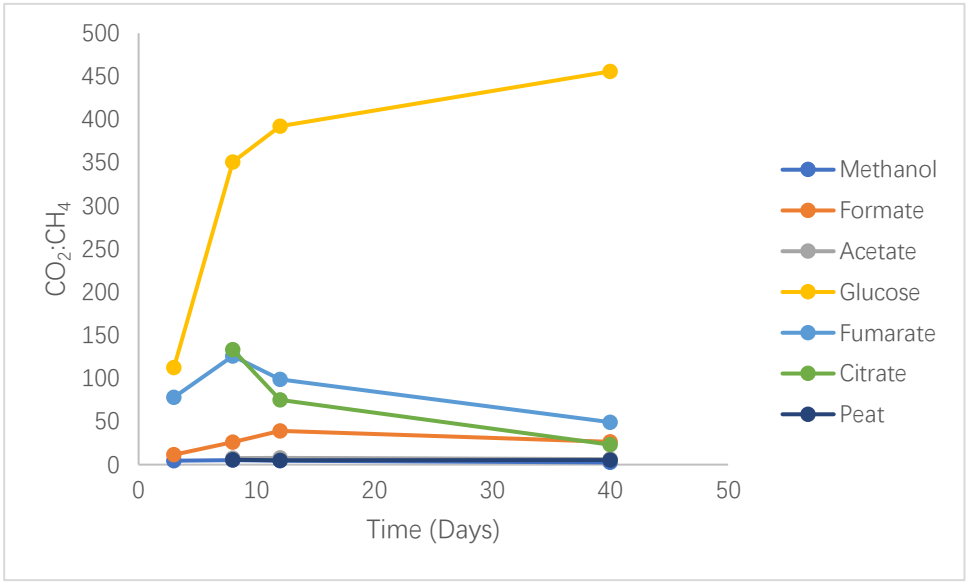



Figure 6: Greenhouse gas production in microcosms amended with various carbon substrates: (a) $CO_2$ accumulation with time with different treatments (b) $CH_4$ accumulation with time for different treatments. (c) $CO_2$:$CH_4$ ratio with time with different treatments

### 4.5 The Limitation of Greenhouse Gas Production by pH

The ambient pH for peatland environments tends to be acidic. For example, porewater pH in the SPRUCE peatland ranges from 3 to 4 (Lin et al., 2014). The question addressed in these experiments is whether ambient pH limits microbial metabolism, and specifically greenhouse gas production, in the SPRUCE peatland? Previous research showed that lower pH can significantly suppress methanogenesis and other microbial activities in peatlands (Dise & Verry, 2001). Conversely, other studies showed that elevated pH would have a positive effect on methanogenesis in certain peatlands within an acceptable range (Ye et al., 2012). What is the optimum pH condition for microorganisms especially methanogens to be the most active? What is the upper limit of pH for normal functioning of microorganisms? Is there a threshold below which pH will suppress methane production in S1 bog peat soils? In addition to these questions, the objective was to determine the optimal pH condition for the cultivation of novel anaerobic microorganisms from S1 bog peat. To answer

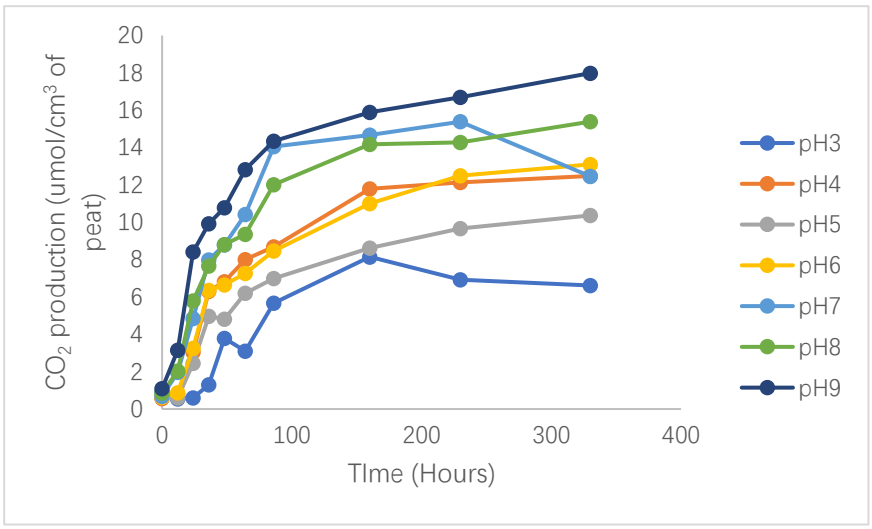
address these questions, the experiment was designed as follows. A 10 times diluted R2A medium was prepared in 100 ml porewater and pH was adjusted to 3, 4, 5, 6, 7, 8 and 9, respectively with 1M HCl or 1M NaOH and then filter sterilized with 0.22 um filters. The remaining procedure was similar to that described above for the substrate utilization experiment. Peat was added as the inoculum for this experiment. Triplicates were prepared for each pH condition. An uninoculated control, was prepared for each pH condition to determine the effects of chemical processes along with microbial metabolism on gas production (Supplemental Figure A4). Kinetics were calculated based on gas accumulation during the exponential phase of microbial growth and gas production. Thus, 12 to 36 hours was selected as the time period for calculation of gas production rates. Greenhouse gases were quantified in the headspace of culture vials by GC analysis at 0, 12, 24, 36, 48, 60, 80, 120, 158 hours in the first week of incubation, once per day in the second week and 2 to 3 times per week afterward. Results are shown in Figure 7.

The pH of the medium in all cultures was checked at days 0 and 14. Results are shown in Table 2.

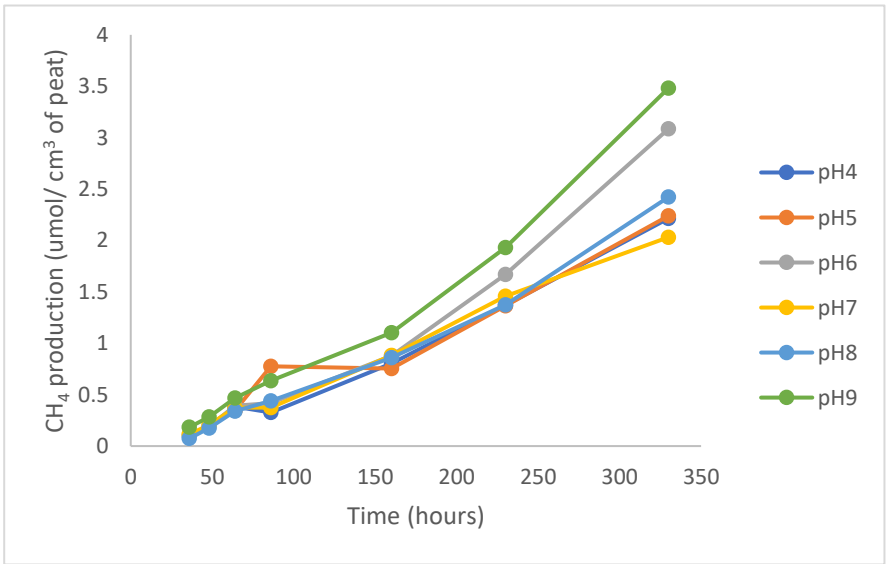
Table 2: Comparison of measured pH values between 0 and 14 days of incubation.

| Initial pH | pH at day 14 |
|---|---|
| 3 | 3.5 |
| 4 | 4.5 |
| 5 | 4 |
| 6 | 5.2 |
| 7 | 5.8 |
| 8 | 5.9 |
| 9 | 6.6 |

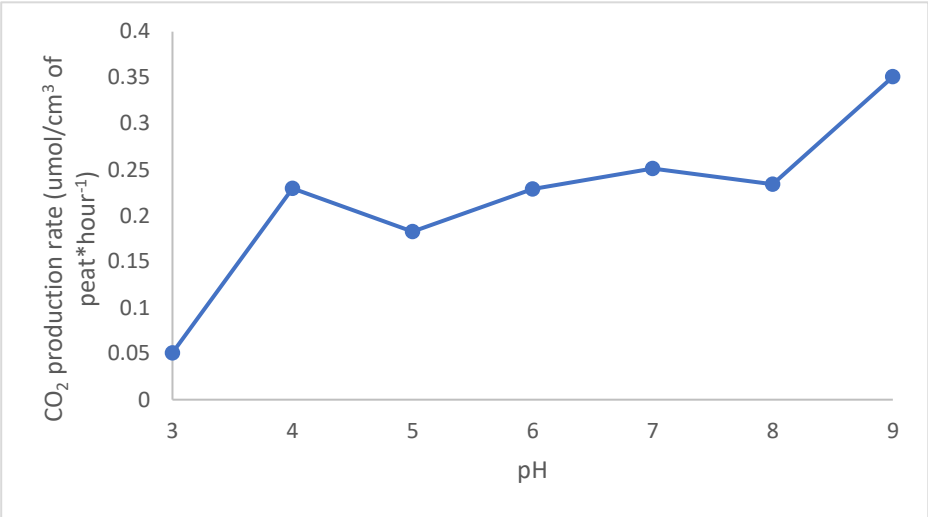
(a)


20
18
16
14
12
10
8
6
4
2
0
0
100
200
300
400
CO2 production (umol/cm3 of peat)
TIme (Hours)
pH3
pH4
pH5
pH6
pH7
pH8
pH9

(b)


4
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
300
350
CH4 production (umol/ cm3 of peat)
Time (hours)
pH4
pH5
pH6
pH7
pH8
pH9

(c)




(d)

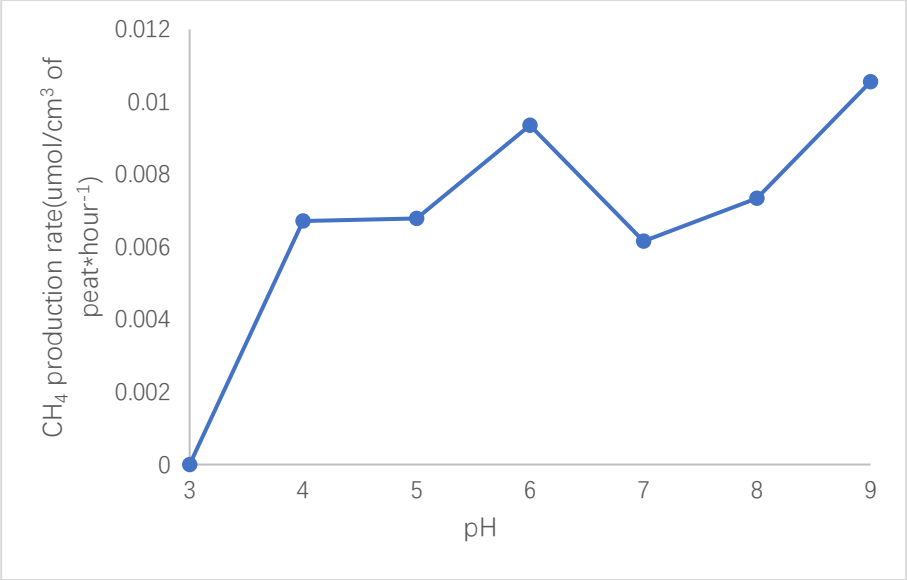


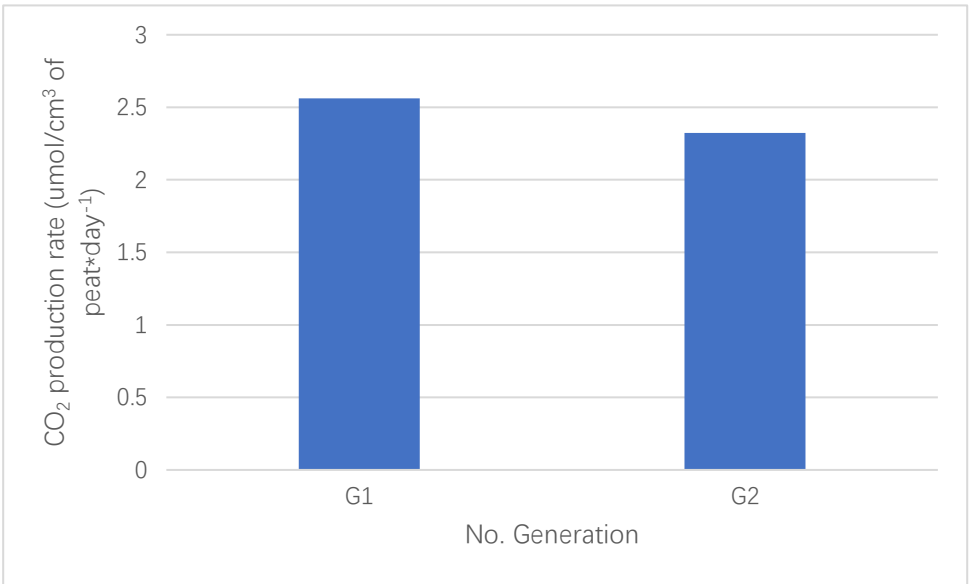

Figure 7: Greenhouse gas production in cultures adjusted to a range of initial pH: (a) $CO_2$ accumulation with time in different treatments (b) $CH_4$ accumulation with time for different treatments. (c) $CO_2$ production rate determined from the linear phase of production (d) $CH_4$ production rate determined from the linear phase of production.

### 4.6 Enrichment of Anaerobic Microorganisms from Peat and Porewater

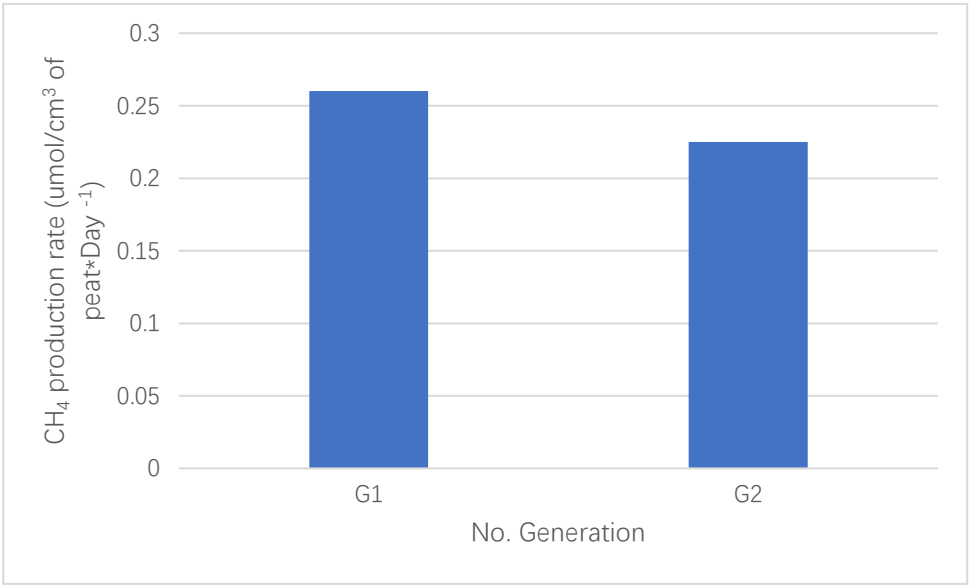
Based on the results from earlier experiments described above in section 4.3 on reducing agents, a revised protocol was developed for the enrichment of anaerobes from peat and porewater. From the reductant experiments, it appeared the citrate from TiCitrate was being fermented. Thus, Ti(III)Nta was chosen as the reductant for subsequent transfers of the enrichment cultures. Four treatments were conducted for transfer in R2A medium. The inocula and treatments were peat + TiNta, peat only, porewater + TiNta, and porewater only. Although a 10 % inoculum of each culture was transferred, even less peat could be transferred due to the pore size limitation of the needles. Here the relative activity between transfers was calculated according to gas production rate. Results are presented in Figure 8.

The pH of each culture was also measured at day 30 with the following results: Peat + TiNta, 5.4; Porewater + TiNta, 5.3; Peat only, 4.4; Porewater only, 4.4.

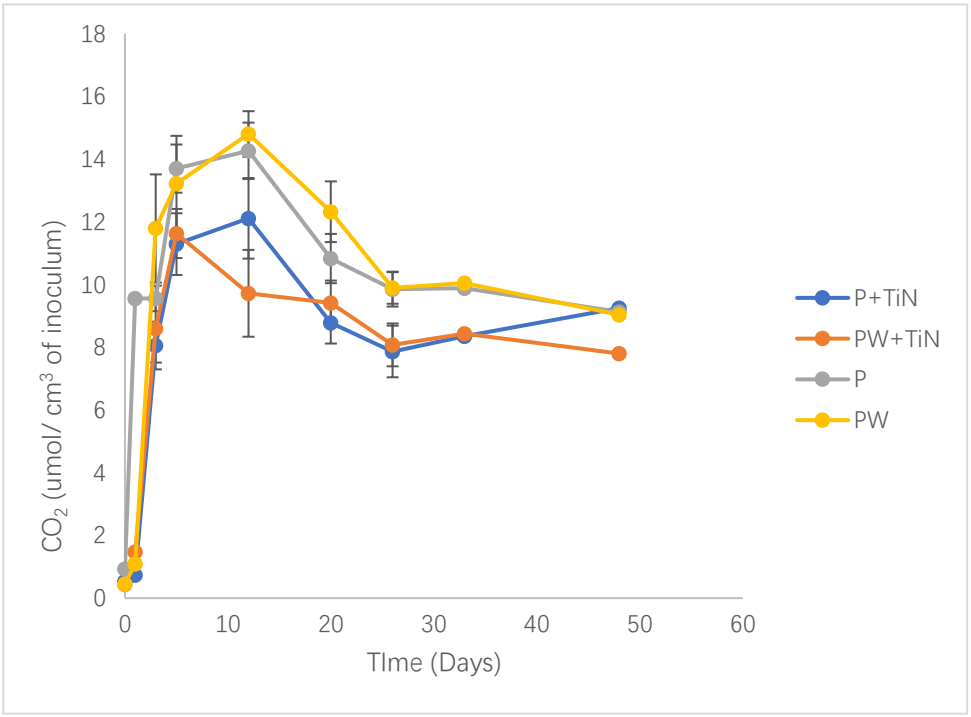
(a)



(b)


0.3
0.25
0.2
0.15
0.1
0.05
0
CH4 production rate (umol/cm3 of peat*Day -1)
G1
G2
No. Generation

(c)


18
16
14
12
10
8
6
4
2
0
CO2 (umol/ cm3 of inoculum)
0
10
20
30
40
50
60
TIme (Days)
P+TiN
PW+TiN
P
PW

(d)

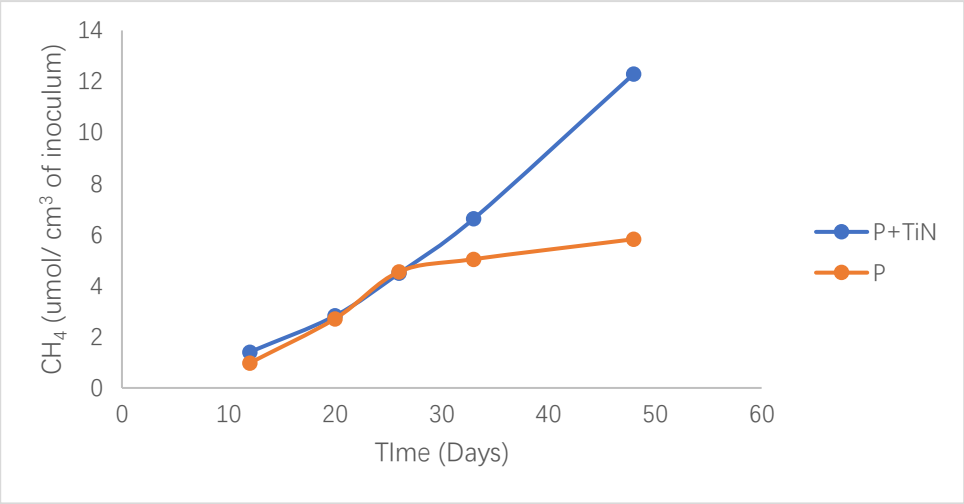
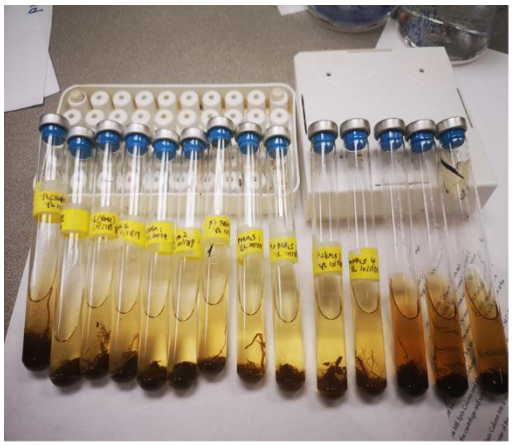



Figure 8: Summary of greenhouse gas production in enrichment cultures (a) $CO_2$ production rates calculated for transfers 1 and 2, (b) $CH_4$ production rates calculated for transfers 1 and 2 based on enrichment cultures inoculated with peat and treated with Ti(III)Nta; (c) $CO_2$ accumulation with time (d) $CH_4$ production with time during transfer 2 according to treatment.

(a)



From left to right: 1-4 Peat+0.83mM Ti(III) Citrate.
5-7 peat+0.83mM Ti(III) Nta
8-10 Peat+1mM Na2S
11-13 Peat+autoclave 3 times


From left to right (Photographed in dark)
1-3 Porewater+0.83mM Ti(III) Citrate
4-7 Porewater+0.83mM Ti(III) Nta
8-10 Porewater+0.83mM Na2S
11-13 Porewater+Autoclave three times

(b)

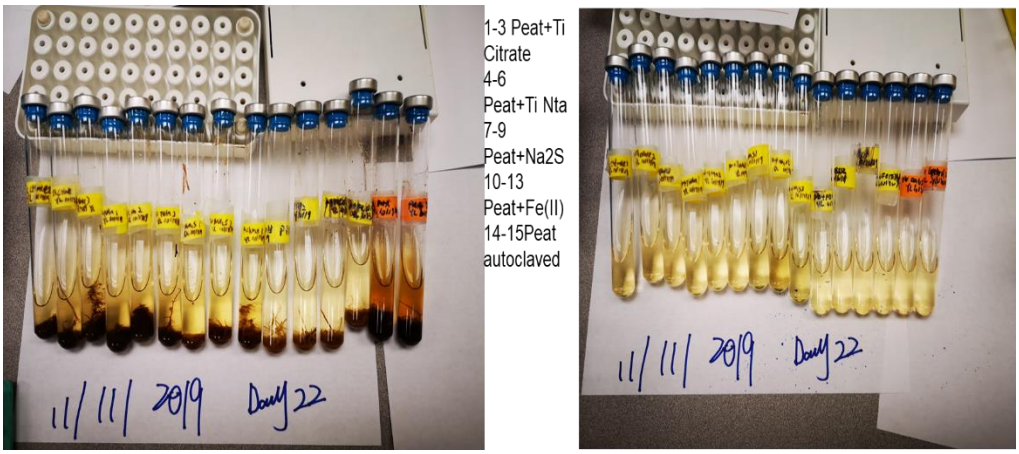



Figure 9: Enrichment cultures passage 1 shown at (a) Day6 and (b) Day 22. Treatments are annotated in the legends of each image.

### 4.7 The Transformation of Organic Substrates in Peat Microcosms

Microcosms initiated in section 4.4 were analyzed for organic acids. Microcosms were sampled at day 0, 12, and 25. Each time a 1 ml sample was taken from the microcosm, 1 ml porewater medium was added back to the culture to avoid a drastic change of incubation conditions. Results are shown in Table 3 and the original ion chromatographs are presented in supplemental Figure A3.

Table 3: Organic acids detected in peat microcosms amended with different carbon substrates.

| Treatment / Time | Day 0 | Day 12 | Day 25 |
|---|---|---|---|
| 5 mM Acetate | (1) 6.3 mM Acetate | (1) 6.5 mM Acetate | (1) 7.7 mM Acetate |
| 5 mM Citrate | (1) 2.8 mM Citrate | (1) 2.4 mM Citrate<br>(2) Unknown fatty acid 1<br>(3) 0.16 mM Propionate | (1) 5 mM Acetic<br>(2) Unknown fatty acid 1<br>(3) 0.13 mM Propionate |

| Table 3: Continue | | | |
|---|---|---|---|
| 10 mM Formate | (1) 2.7 mM Formate | (1) 2.9 mM Formate | (1) 1.4 mM Acetate<br>(2) Unknown fatty acid 1 |
| 10 mM Fumarate | (1) 6.4 mM Fumarate | (1) 9.1 mM Fumarate<br>(2) 1.7 mM Lactate<br>(3) 0.11 mM propionate | (1) 1.9 mM Acetate<br>(2) Unknown fatty acid 1<br>(3) 0.4 mM propionate |
| 10 mM Glucose | None | (1) 2.3 mM Acetate<br>(2) Unknown fatty acid 1<br>(3) 1.1 mM Propionate | (1) 7.8 mM Acetate<br>(2) 1.6 mM Lactate<br>(3) 0.1 mM Propionate |
| 10 mM Methanol | None | None | None |
| Peat control | None | None | None |

### 4.8 Transformation of Organic Substrates in the pH Experiment

Cultures prepared in section 4.5 above were also analyzed for organic acids. Cultures were sampled at days 0, 7, and 15 for each pH treatment. In this experiment, the third culture tube of the triplicates contained 12 ml of medium, which was 2 ml larger than the others. Therefore, 1ml samples could be collected over time for organic acid analysis. (However, due to the COVID-19 situation, I was unable to finish this part of experiment)

# Chapter 5: Discussion

## 5.1 Porewater Sampling

Porewater was sampled for characterization of microbial communities using cultivation-based along with cultivation-independent molecular techniques. This thesis focused on cultivation-based approaches. Nonetheless, I participated in the successful filtration of porewater for molecular analyses. It was hypothesized that the time needed for filtration would be positively correlated with microbial abundance in the sample. This appeared to be the case as porewater samples from the shallowest and deepest depths required the most and least amount of time for filtration, respectively, in agreement with previous estimates of microbial abundance in peats collected from the S1 bog (Lin et al., 2014). However, lower abundance does not necessarily less important. Microbes in the deep peat are exposed to an extreme environment from which novel microorganisms or interesting genes or enzymes may be discovered. Microorganisms in deep and shallow peats likely carry out very different functions so that the significance of investigation at any depth cannot be underestimated (Cadillo-Quiroz et al., 2006). Most importantly, the deep peat environment is rapidly changing with climate, and microbes found there may hold the key to understanding the fate of ancient carbon stored there. Detailed information about microorganisms at the different porewater depths will be revealed by metagenomic analysis that is currently underway.

## 5.2 Pre-incubation

Pre-incubation experiments were conducted to determine baseline rates of metabolism, as greenhouse gas production, in unamended porewater and peat soils. Baseline rates were low in porewater collected in August of 2019, with little to no methane production and minor respiration activities observed as $CO_2$ production.

Even after porewater was amended with millimolar amounts of acetate, methane production was not detected for a month, indicating that the porewater samples not limited by carbon substrates. In fact, minor $CH_4$ production was detected after almost three months of pre-incubation, only from the surface depth (25 cm) and at higher incubation temperatures (14 °C). One explanation for these results could be that microbial metabolism is limited by the supply of electron acceptor. Microbial communities could be present in such low abundance or adapted to such specialized substrates that metabolism of amended acetate is slow. In addition, another parameter such as redox potential or pH may be limiting activity in the laboratory. In an unpublished work done by other members of our lab, benzoate and glucose was also showed little to no stimulation of $CO_2$ and $CH_4$ production. However, when fumarate was added, $CO_2$ but not $CH_4$ production was significantly stimulated. This may indicate that electron acceptor is the limiting factor of respiration and fumarate is fermented to $CO_2$ in this system.

### 5.3 Reducing Agents

Reducing agents were studied in part because of limited activity observed in the porewater incubations described above. Four different reducing agents were applied in this experiment to determine the optimal redox potential as well as to create better conditions for the cultivation of target anaerobic microorganisms. Resazurin is a redox potential indicator. Based on the time required to observe a color change (clearing) from different reductant solutions, it could be inferred that reductants containing Ti(III), no matter in the form of Ti(III) Citrate or Ti(III) Nta had the highest capability to lower the redox potential, not only because of the speed of clearing, but also that the concentration of Ti(III) added was the lowest among all candidate reducing agents. Dissolved sulfide is often used as a reductant for the cultivation of methanogens(Oremland, Marsh, & DesMarais, 1982; Stieb & Schink, 1989). Sulfide was determined to be the second most effective of the reductants tested, while Fe(II) was the least effective. Iron(II) was added at the highest

concentration but took the longest to clear.

Greenhouse gas production provided a proxy for the metabolism of organic matter in the presence of different reducing agents. Rates of $CO_2$ production were generally higher in peat as compared to porewater incubations, further confirming that microbial activity is higher in peat. However, Ti(III) citrate stimulated the highest rates of $CO_2$ production observed, whether in porewater or in peat. In addition, similar amounts of $CO_2$ were produced in porewater as compared to peat amended with Fe(II), albeit with a longer lag time. The results show that sufficient microorganisms are present in porewater that are capable of organic matter degradation, however, these organisms are only active under certain conditions. While Ti(III) served as an effective reductant to lower redox potential, activity was more limited by substrate, with citrate serving as a favored substrate by microorganisms in peat or porewater. Both redox potential and substrate addition likely contributed to the rapid $CO_2$ production observed. In contrast, other reducing agents did not show a major effect on carbon metabolism. TiNta and sulfide were observed to have a minor effects on $CO_2$ production in peat, whereas in porewater, these reductants inhibited overall degradation activity. The results also point to a limitation by electron acceptor in the incubations. The majority of CO2 was produced during the first two weeks of incubation, and the CO2/CH4 ratio declined in parallel, in peat incubations. This suggests that alternate electron acceptors stimulated respiration over methanogenesis until they were depleted in the incubations.

Methanogenesis showed a very different response to reducing agents in comparison to overall $CO_2$ production. Similar to observations in the pre-incubation experiments, little to no methane was produced in porewater, indicating that redox potential is not likely limiting methanogenesis there. Methanogen are either inactive or not present in porewater collected in Summer 2019. Only Ti(III) Nta stimulated methane production in peat incubations and this could be due to the elevated pH (6.4-6.5) observed after two weeks in these cultures. Based on these results, Ti(III)Nta was chosen as the reducing agent for enrichment culture transfers.

## 5.4 Substrate Utilization in Peat Microcosms

Another major objective of this thesis research was to investigate the carbon substrates supporting greenhouse gas production in the S1 bog. These microcosm experiments differed from enrichment cultures in that no complex carbon or nutrients were added. Surprisingly, unlike most other soils in which methanogenesis has been studied, activity was not affected by the addition of substrates known to stimulate their metabolism, such as acetate, methanol, and formate. Classic fermentation intermediates, acetate and methanol, also did not enhance overall heterotrophic activity measured as $CO_2$ production. Only fermentable substrates (glucose, citrate, fumarate) and formate were shown to stimulate anaerobic metabolism.

Glucose is a common carbohydrate monomer supporting anaerobic degradation and so it is not a surprise the it stimulated the production of $CO_2$. There are at least two possible mechanisms by which fumarate can play a role in $CO_2$ production. Fumarate serves as an electron acceptor for anaerobic respiration (Asanuma, Iwamoto, & Hino, 1999; Butler et al., 2006; Kröger, 1974; Tielens & Van Hellemond, 1998) and it may also be fermented to acetate, formate or propionate (Stieb & Schink, 1984). Citrate was employed based on our previous observations that Ti(III) Citrate stimulated rapid $CO_2$ production.

Organic acids were analyzed in the microcosms to provide further evidence for the operative metabolic pathways. Acetate was not utilized in agreement with the lack of stimulation of greenhouse gas production. Although methanol was not measured, gas production was also not enhanced with this substrate. Formate, in the form of formic acid, was not utilized by microorganisms in peat, which matched the gas production data obtained. However, when sodium formate was added as a carbon substrate, it was found that formate could be utilized and acetate was produced. Therefore, it is likely that acetogens outcompeted methanogens to convert formate, which was considered to be a hydrogen-like compound, to acetate (Stams & Dong,

1995).

Citrate was shown to be a favored substrate of peat microorganisms. Citrate, which stimulated the largest amount of $CO_2$ and $CH_4$ production, appeared to be fermented mainly to acetate. Glucose, which enhanced $CO_2$ but not methane production, was fermented to acetate, propionate, and lactate. Fumarate appeared to be fermented to lactate and acetate, while supporting both $CO_2$ and $CH_4$ production. However, it is still not clear from this analysis whether it could serve as an electron acceptor. Further research would help to determine the function and role of fumarate as a carbon substrate in peat soils. An unknown peak with a retention time of 16 minutes appeared multiple times in cultures incubated with glucose, fumarate, and formate, suggesting that it represents a fairly common fatty acid intermediate. Therefore, future research should attempt to identify this intermediate.

Overall, it could be verified that peatland soils produce much more $CO_2$ than methane. Surprisingly, methanogenesis was stimulated by citrate and fumarate but not by glucose. This result showed that there is not necessarily a direct correlation between microbial fermentation and respiration. It is concluded that the terminal steps in organic matter degradation, respiration reactions, are decoupled from fermentation in soils of the S1 bog. Respiration reactions appear to be finely tuned to specialized substrates produced in peat soils, and these substrates have yet to be defined. One possibility is that hydrogen, produced from the fermentation of citrate and fumarate, is driving methanogenesis. Syntrophy is considered to provide substrates to support methanogenesis (McInerney et al., 1981). However, in the S1 bog, this relationship appears to be disconnected. One explanation could be that methanogen activity in peat is already saturated, even though syntrophs are very active. The addition of microbial genomics data would help to interpret the various metabolic pathways implicated here.

### 5.5 Effect of pH on Gas Production

Two successive experiments targeted the effects of pH on methanogenesis in

this thesis. In the first experiment, a pH range of 4 to 7 was selected and the hypothesis was that the optimum pH for microbial metabolism, especially for methanogens, should be between 6 to 7. However, several problems were encountered when analyzing the results for this experiment. First, similar rates of $CH_4$ production were observed in all treatments and so an optimum pH was not evident. This was likely due to the fact that methanogenesis was so rapid in all treatments because R2A was added so that sufficient substrates and conditions were provided at the beginning, such that the time course did not capture differences in the rates of different treatments. In addition, the pH range tested was insufficient and should be expanded.

The second experiment was optimized by adjusting the time course to collect more time points in the first few days as well as by expanding the pH range of treatments. The results showed that both $CO_2$ and $CH_4$ production data were optimal at the highest pH tested. Generally, the results supported the hypothesis that higher pH will result in higher $CH_4$ production. Also, $CH_4$ and $CO_2$ production were severely inhibited below pH 4, indicating that this pH represents a threshold below which microbial metabolism is restricted. In both pH experiments, it was observed that $CO_2$ production declined under all pH conditions at approximately day 3, suggesting that respiration or fermentation activities were saturated fairly quickly. In contrast, $CH_4$ production rates were maintained even after a month. This result could be interpreted as methanogens require a longer doubling time for growth, and they utilize substrates and nutrients provided by their syntrophic partners over a longer period of time. For example, the doubling time of *Methanosaeta* spp. was estimated at up to 9 days (Anderson, Llamedo, Tohidi, & Burgass, 2003), which is slow in comparison to bacteria that may inhabit in this environment, with doubling times of minutes to hours (Li et al., 2015; Pecoraro, Zerulla, Lange, & Soppa, 2011; Shepherd et al., 2010). However, it is still significant to obtain genomic information and analysis under different pH conditions so that more information is uncovered on how pH alters microbial genomics in S1 bog.

## 5.6 Enrichment

A major objective of this thesis research was to cultivate and enrich anaerobic microorganisms from peatlands, since so few of these microbes have been cultivated in the past (Dedysh, 2011). This objective was clearly achieved. The metabolic activity of enrichment cultures, as indicated by $CO_2$ and CH4 production, was rapid and did not diminish between culture transfers. It was concluded that TiNta is the most effective reductant to support the growth of anaerobes from S1 bog porewater and peat. In the second transfer, the TiNta treatment showed the highest rates of $CH_4$ production, indicating that methanogens were active and enriched over the controls. However, an elevated pH was also measured in the TiNta treatment relative to the controls. The buffering of pH remained a challenge throughout the thesis research. Future studies should add a pH buffer, such as Homopipes, to the enrichment cultures and then repeat the experiment to determine whether TiNta continues to stimulate the metabolism of anaerobes in this system. The hypothesis that porewater collected in August, 2019, could not support the growth of methanogens was rejected since methanogenesis was sustained even though very little of the original peat remained in the culture. Growth was evident as elevated turbidity in comparison to uninoculated controls. Cell counts should be conducted to verify if microorganisms were indeed enriched. The enrichment culture medium employed here successfully enriched targeted organisms, or at least maintained their growth. Soil-free enrichments were obtained that will enable further studies of the physiological ecology of anaerobic prokaryotes in peatlands.

## Chapter 6: Conclusions and Significance

Very few anaerobic prokaryotes have been cultivated from peatlands (Dedysh, 2011). In this thesis, anaerobic heterotrophs were enriched using cultivation-based methods and shown to mediate the rapid metabolism of organic matter under strictly anoxic conditions. Knowledge was gained on the physiological controls of anaerobes in peat soils. Rapid production of CO2 and CH4 were observed in the presence of complex carbon. Although redox potential and pH were also shown to have an impact, the results of this thesis showed that organic carbon substrates, and potentially electron acceptor availability, have a larger influence on anaerobic heterotrophic activity. Porewater was explored as a model system for studying anaerobic heterotrophs of S1 bog. However, methanogenesis was slow and unpredictable in porewater under the conditions tested. Thus, peat soil was shown to be a better model system for the study of anaerobic metabolism at S1. Unlike most other soils, methanogen activity was not affected by the addition of substrates known to stimulate their metabolism, such as acetate, methanol, and formate. These classic fermentation intermediates also did not enhance overall heterotrophic activity measured as CO2 production. Only fermentable substrates (glucose, citrate, fumarate) were shown to stimulate anaerobic metabolism. It is concluded that the terminal steps in organic matter degradation, respiration reactions, are decoupled from fermentation in soils of the S1 bog. Respiration reactions appear to be finely tuned to specialized substrates produced in peat soils, and these substrates have yet to be defined. Overall, it could be verified that peatland soils produce much more CO2 than methane. Knowledge gained on the characteristics of the peatland microorganisms can be used to direct isolation of pure cultures for further physiological studies. Freshwater wetlands such as peatlands serve as important sources of greenhouse gases on a global scale. In order to better predict the response of greenhouse gas emission to climate change, the physiology and environmental controls of anaerobic microorganisms in soils must be further understood. This thesis work represents a

substantial step in the direction of understanding the physiology of anaerobic heterotrophs in peatland soils.

## Chapter 7: Future Research

In nearly all of the incubation studies in this thesis, the CO2:CH4 ratio declined with time in the incubation, indicating that overall heterotrophic activity was likely limited by the availability of electron acceptors. Therefore, future research should focus on the terminal electron accepting pathways in S1 bog soils, since electron acceptors are likely to regulate the emission of greenhouse gases. As previously introduced, common inorganic electron acceptors such as sulfate, Fe(III), nitrate are lacking in peatlands. Possible alternative electron accepting pathways could involve the respiraton of organic chemicals. Peat soils are rich in organic matter and especially lignin-derived compounds produced by Sphagnum peat mosses. Similar to the cow rumen, which is also rich in organic matter and depleted in minerals, organic compounds such as monensin could serve as electron sinks (Callaway & Martin, 1996; Fonty & Morvan, 1996; R. M. Wilson et al., 2017). Fumarate, as tested already, is representative of the simplest form of organic compounds that could serve as an electron acceptor (Lin et al., 2015). Some more complex carbons such as quinone, and quinone-derived compounds such as napthaquinone could also serve as efficient electron sinks during anaerobic degradation processes (Cervantes et al., 2001; Cervantes, Van Der Velde, Lettinga, & Field, 2000; Tielens & Van Hellemond, 1998), which may significantly impact the production of $CH_4$. Lastly, humic acids, which include a variety of functional groups such as quinone, catechol, phenol, are abundant in soil environments such as S1 bog. If these compounds are efficiently utilized as electron sinks, emission of $CH_4$ would be significantly reduced, resulting in less acceleration of global warming (Bradley, Gaskell, & Gu, 1998). Therefore, organic and inorganic chemicals that could potentially serve as alternative electron acceptors are candidates for upcoming research. The focus should be on figuring out the alternative electron pathways involved in anaerobic degradation and carbon cycles in peatlands.

Another potential area of future research would be the study of methyoxylated

aromatic compounds as substrates for anaerobic metabolism in peat soils of the S1 bog. Recent investigations showed that methoxylated groups are cleaved to produce methane (Mayumi 2016). Also, significant numbers of methoxylated aromatic compounds are derived from the degradation of peat mosses of the genus Sphagnum, which dominate plant communities at S1. It would be interesting to uncover the degradation pathways of methoxy groups under anaerobic conditions. Will the aromatic compounds be degraded by traditional anaerobic degradation pathways? This research could be significant for scientists to construct a more comprehensive model to predict methane emission. In order to better build a model, a more careful collection of information regarding the chemical compositions, especially in terms of methoxylated aromatic compound residues is required from S1 bog. Also, if certain methoxylated compounds were seen to be degraded by microbes, it is necessary to obtain the rate of degradation and the chemical form they are converted to at different timepoints (i.e. intermediates). These compounds may take from days up to several months to be degraded (Mechichi & Sayadi, 2005). Potential methoxylated compounds for study include methoxy-benzoate, cinnamate, phenol, benzene, phenylacetate, benzaldehyde chemicals, vanillin, and benzaldehyde. The degradation pathways could be tracked with high performance liquid chromatography (HPLC) coupled with electrospray ionization-mass spectrometry (ESI-MS) to completely demonstrate the process. Genomic techniques such as metagenomics or metatranscriptomics (Bashiardes, Zilberman-Schapira, & Elinav, 2016) should be combined with HPLC to elucidate the biochemical pathways.

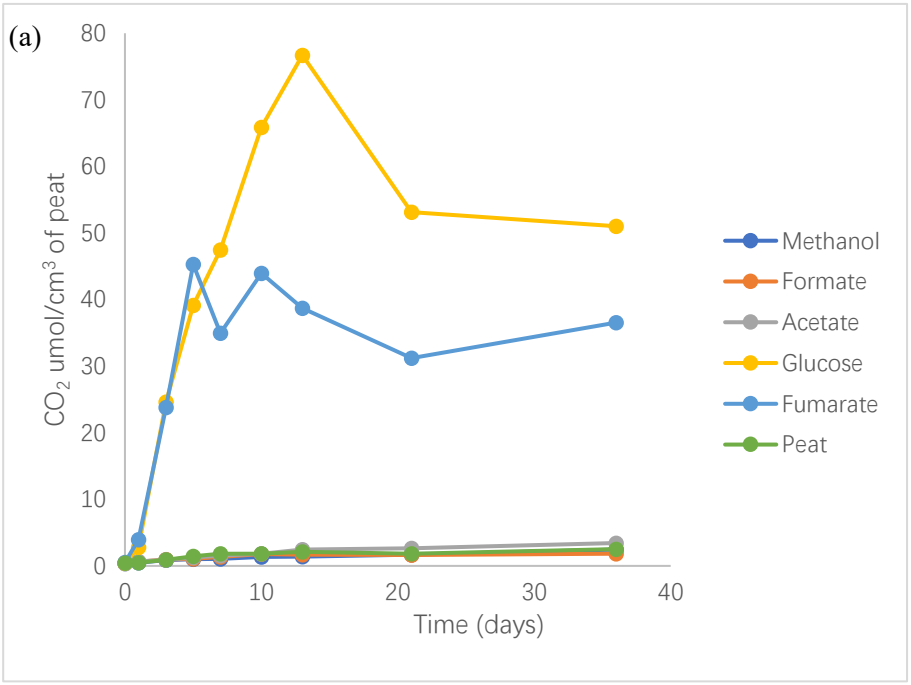
As for the continuation of enrichment of anaerobic microorganisms, The medium (Bräuer, Cadillo-Quiroz, Yashiro, Yavitt, & Zinder, 2006) designed was applied to our project. Cultures in R2A was transferred to Brauer's medium as designed concentration. Successful enrichments were expected to complete after 3-5 passages or even more. Enrichment cultures were supposed to be transfer to fresh medium in the middle of their exponential phase This time point could be determined by GC. Novel microorganisms were planned to be detected by obtaining their 16s

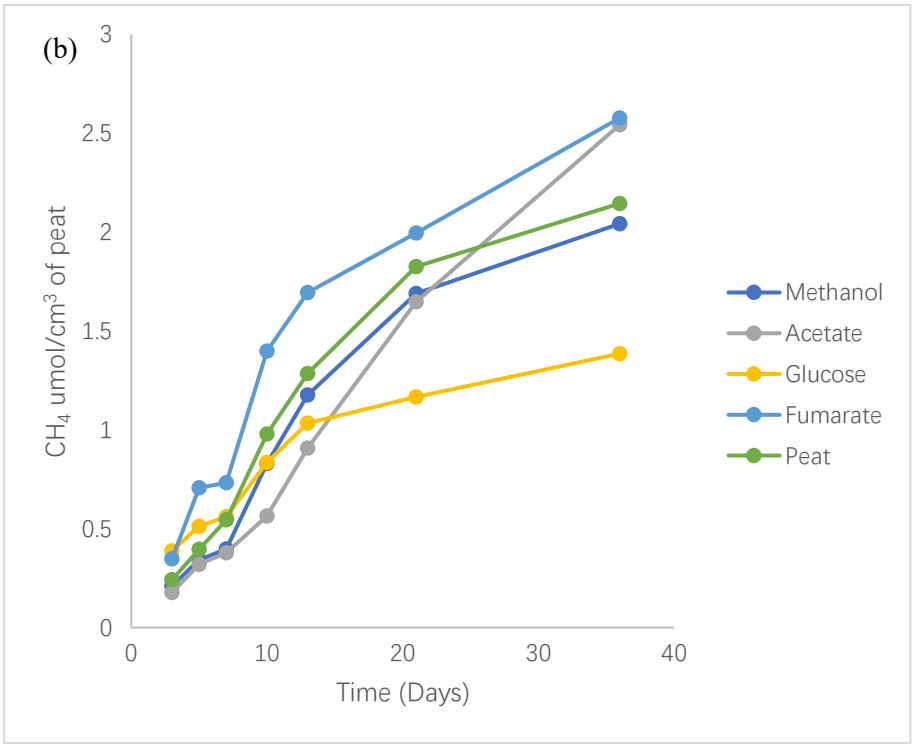
rRNA sequences by PCR amplification and sequencing. To determine novel genes and their abundancy, metagenomics might be a good approach to achieve that.

Isolation of novel microorganisms would be exercised if enrichment succeeded, detailed plan for this part have not been constructed yet, but serial dilution technique in doing isolation may be preferred.

Lastly, the conclusions of this thesis should be further investigated using microbial genomics tools. Hypotheses on the pathways and environmental controls of anaerobic organic matter decomposition should be tested using next generation sequencing of SSU rRNA amplicons and metagenomics. Potential functions revealed in the laboratory could then be explored in field samples. Samples have been archived from this thesis project for future microbial genomics studies. Frozen samples are available for DNA extraction and cultures will be maintained for further investigation. For example, it would be very interesting to determine how pH, reductants and substrates alter microbial community composition. For instance, will the Acidobacteria be more abundant and active at lower pH such as 4 or 5. Additionally, will certain substrates activate syntrophic bacteria that would produce substrates for methanogens (Stams & Plugge, 2009).

## Appendix A: Supplemental Figures



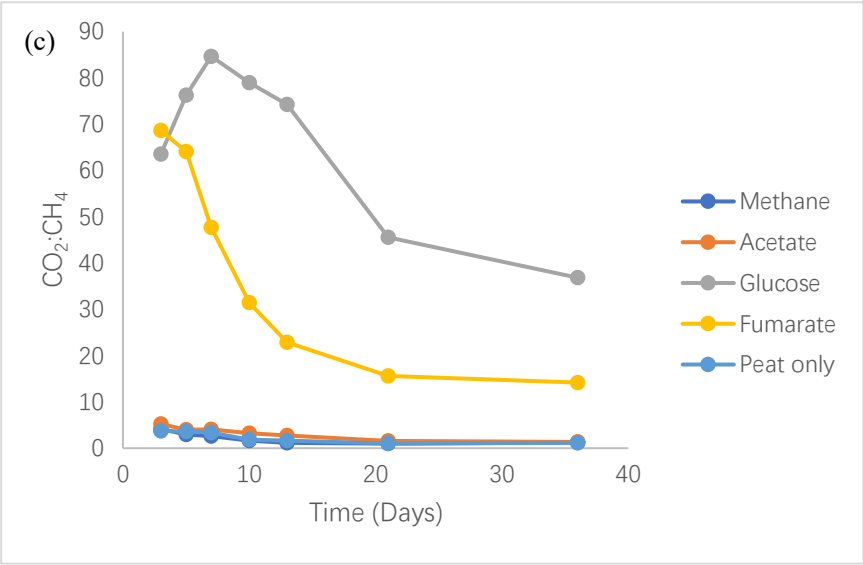
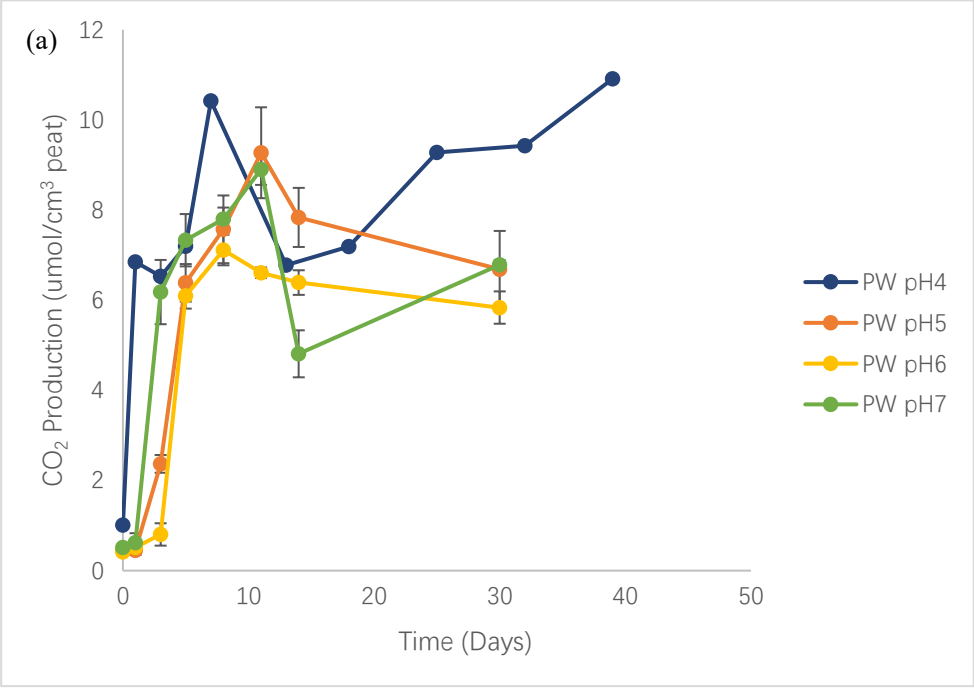
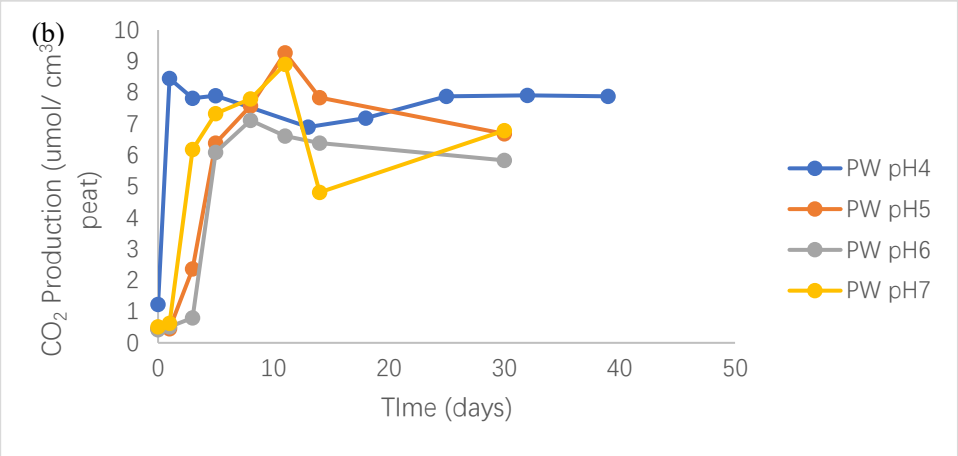
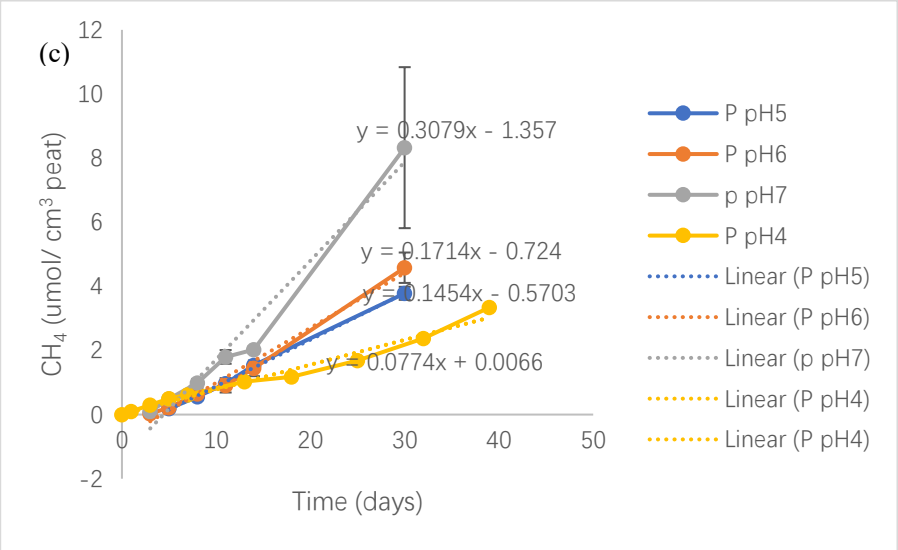
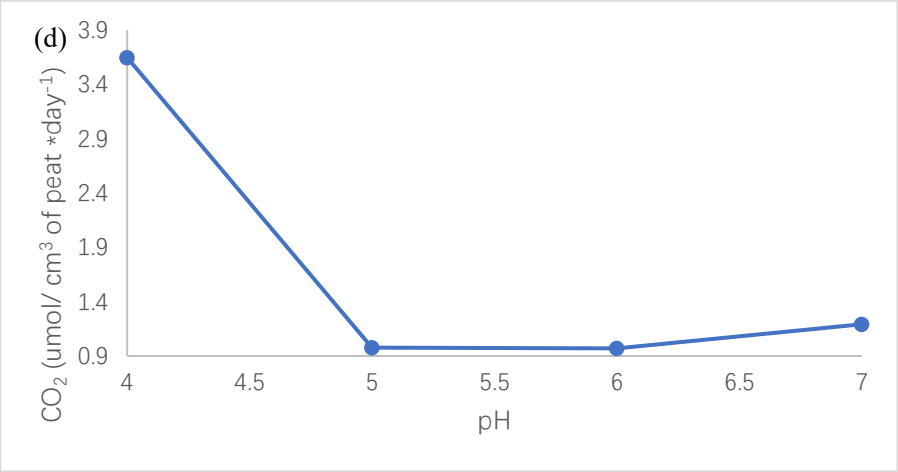


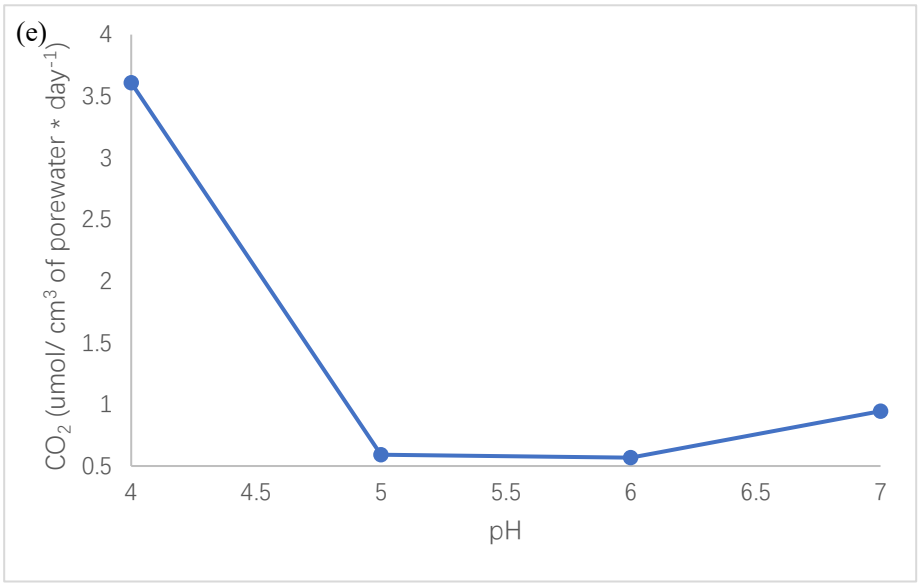

Figure A1: Gas data of peat incubation with different substrates in the first attempt. Figures were plotted with time vs (a) $CO_2$ production (b) CH4 production (c) $CO_2$:CH4 ratio




(b)
CO2 Production (umol/ cm3 peat)
TIme (days)
PW pH4
PW pH5
PW pH6
PW pH7


(c)
CH4 (umol/ cm3 peat)
Time (days)
y = 0.3079x - 1.357
y = 0.1714x - 0.724
y = 0.1454x - 0.5703
y = 0.0774x + 0.0066
P pH5
P pH6
p pH7
P pH4
Linear (P pH5)
Linear (P pH6)
Linear (p pH7)
Linear (P pH4)
Linear (P pH4)


(d)
CO2 (umol/ cm3 of peat *day-1)
pH



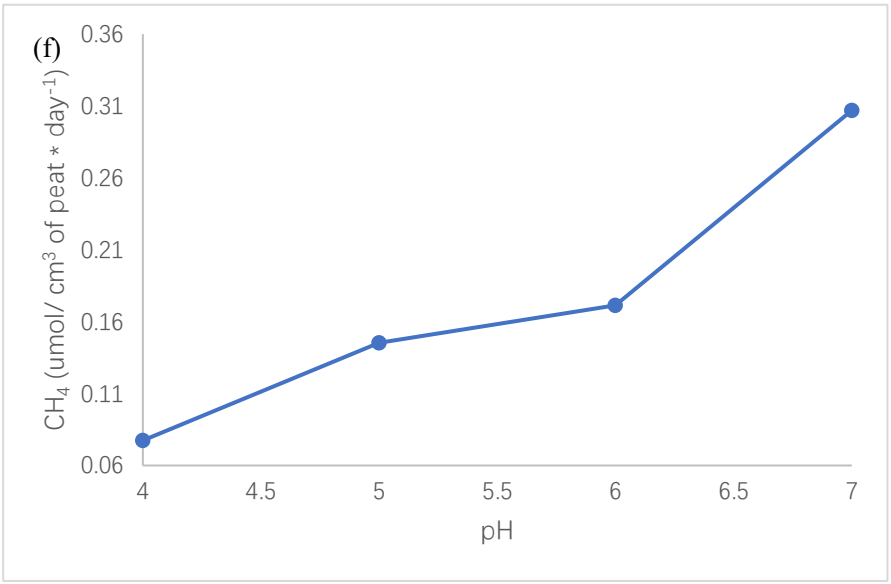
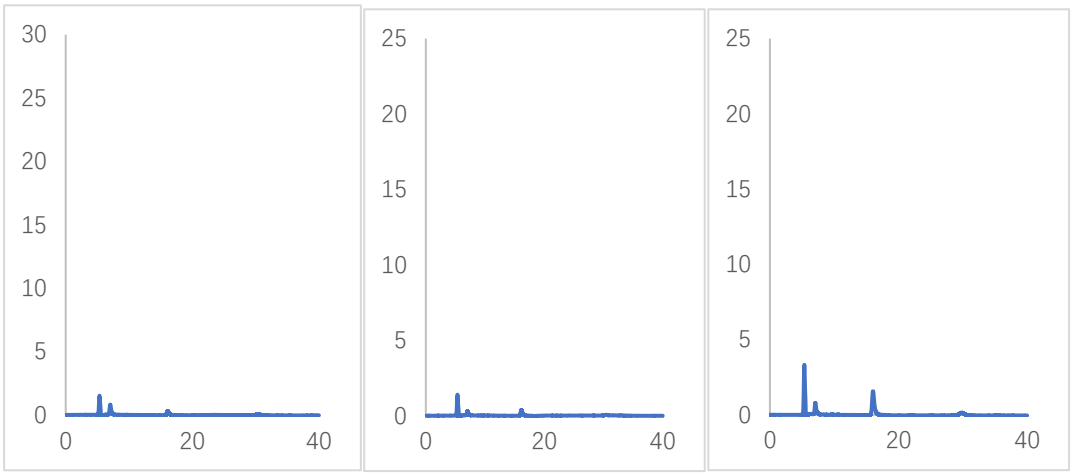




Figure A2: Data from the first attempt of pH experiment. (a) $CO_2$ production over time with peat as inoculum (b) $CO_2$ production over time with porewater as inoculum (c) CH4 production over time and its linear regression formula (d) $CO_2$ production rate in peat inoculated cultures (e) $CO_2$ production rate in porewater inoculated cultures (f) CH4 production rate in peat inoculated cultures.

(a)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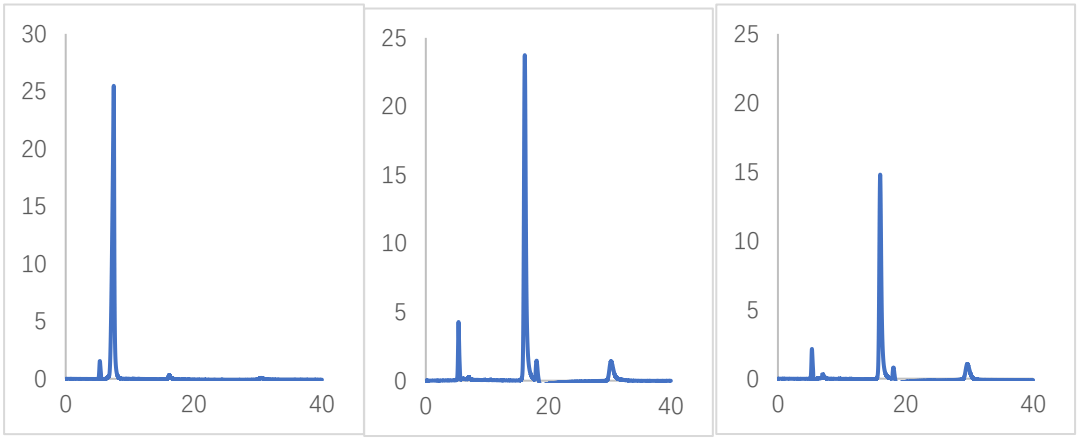
(b)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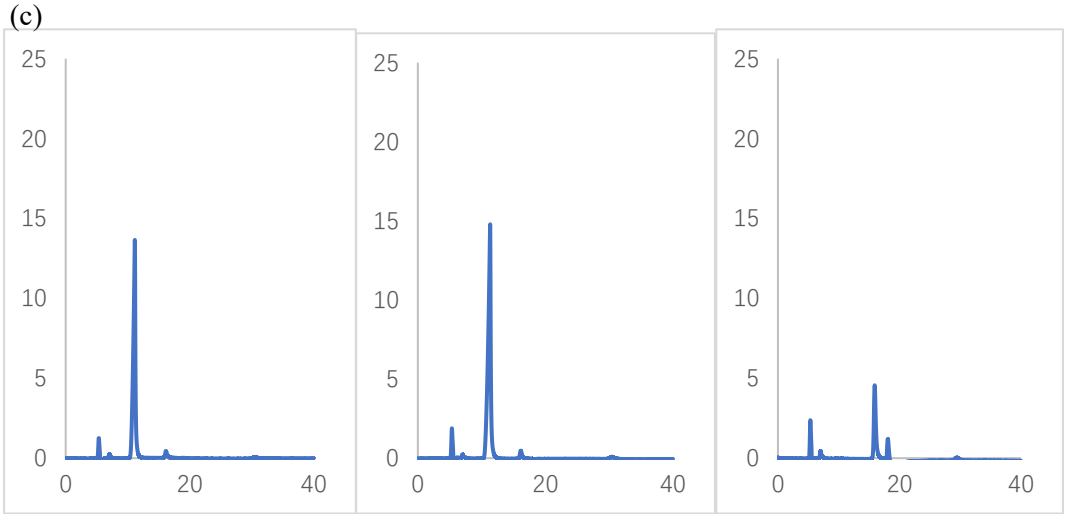
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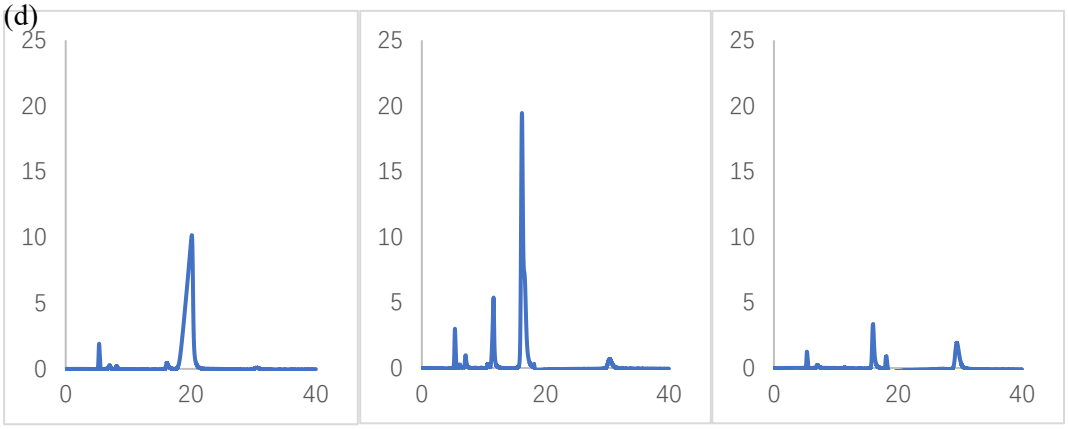
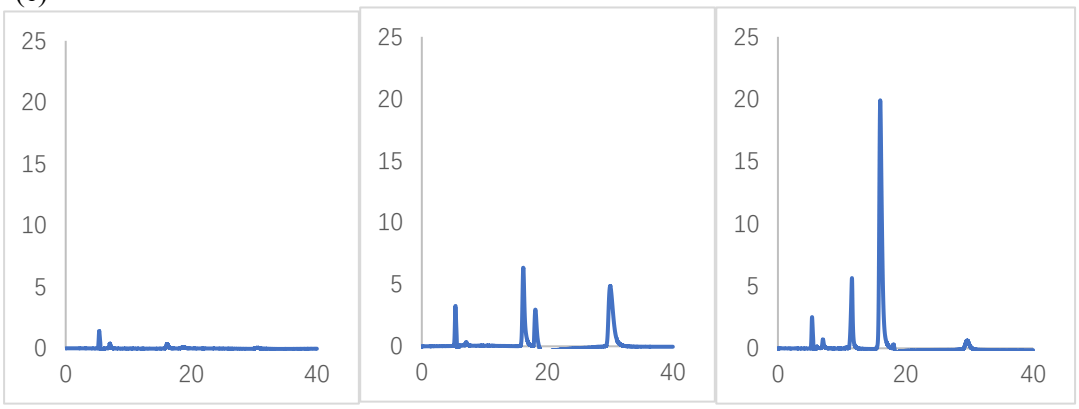
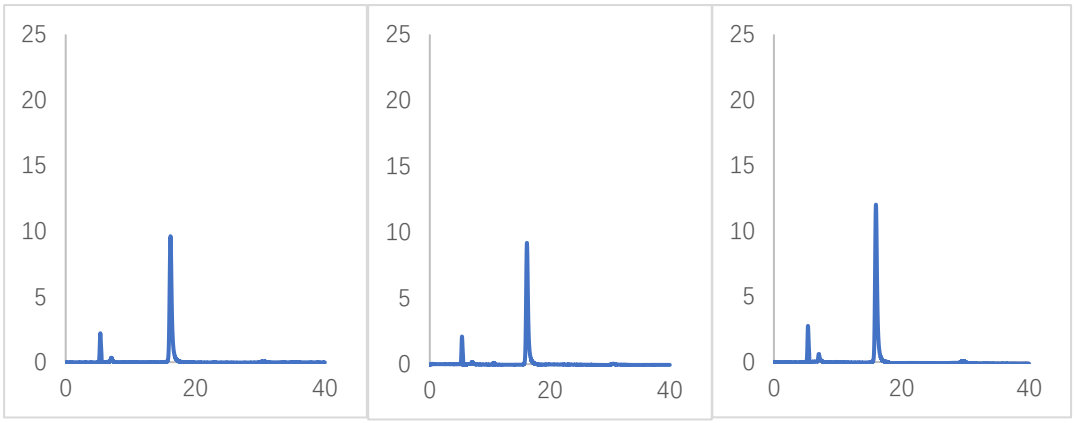
(d)




(e)




(f)



(g)

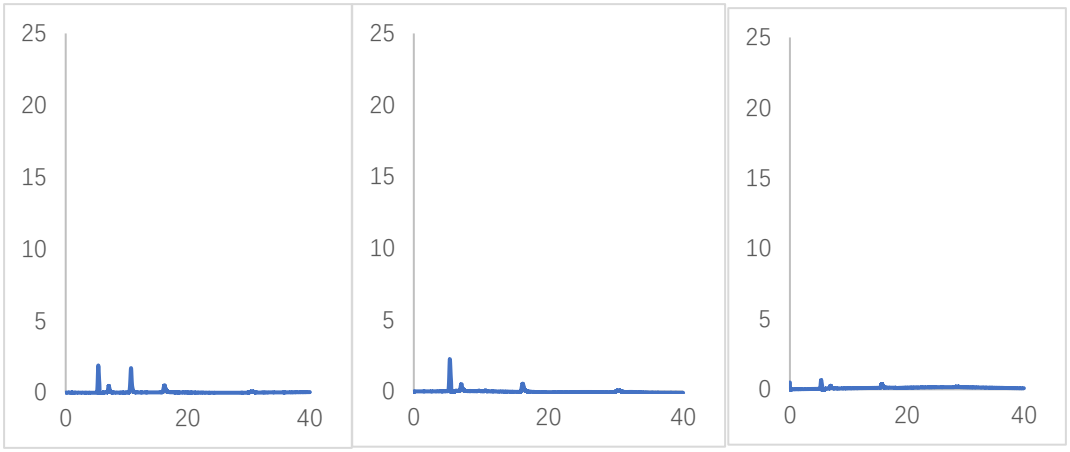



Figure A3: Ion Chromatography graphs. Horizontal axis stands for time in the unit of minutes and vertical axis means conductivity (us). Samples were measured on day 0, 12, 25 for incubation cultures under each treatment (a) Peat only (b) Citrate (c) Formate (d) Fumarate (e) Glucose (f) Acetate (g) Methanol. Every single peak correspond to a retention time stands for a single chemical, which was determined by running its standard solution solely in IC. Peaks appeared at around 5 minutes and 6 minutes are system peaks.

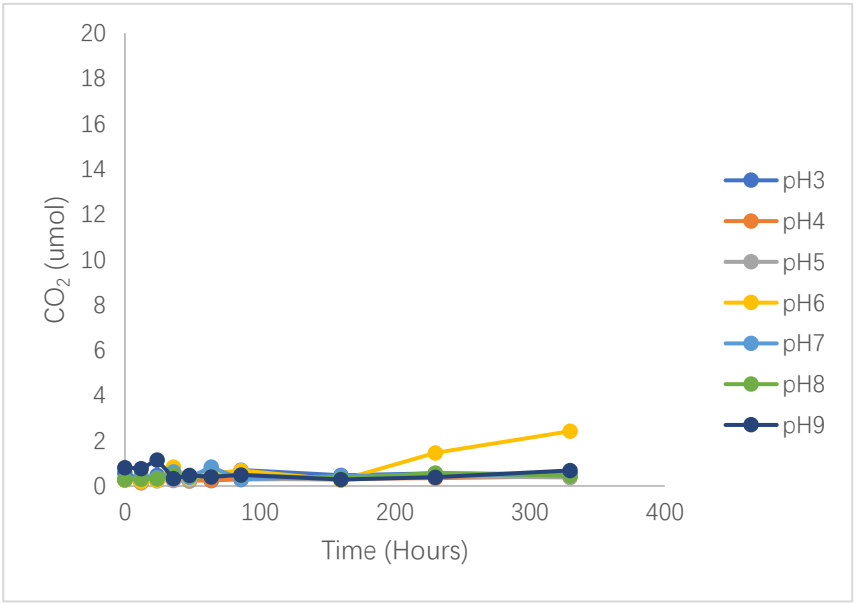



Figure A4: $CO_2$ production over time in blank control cultures at different pHs.

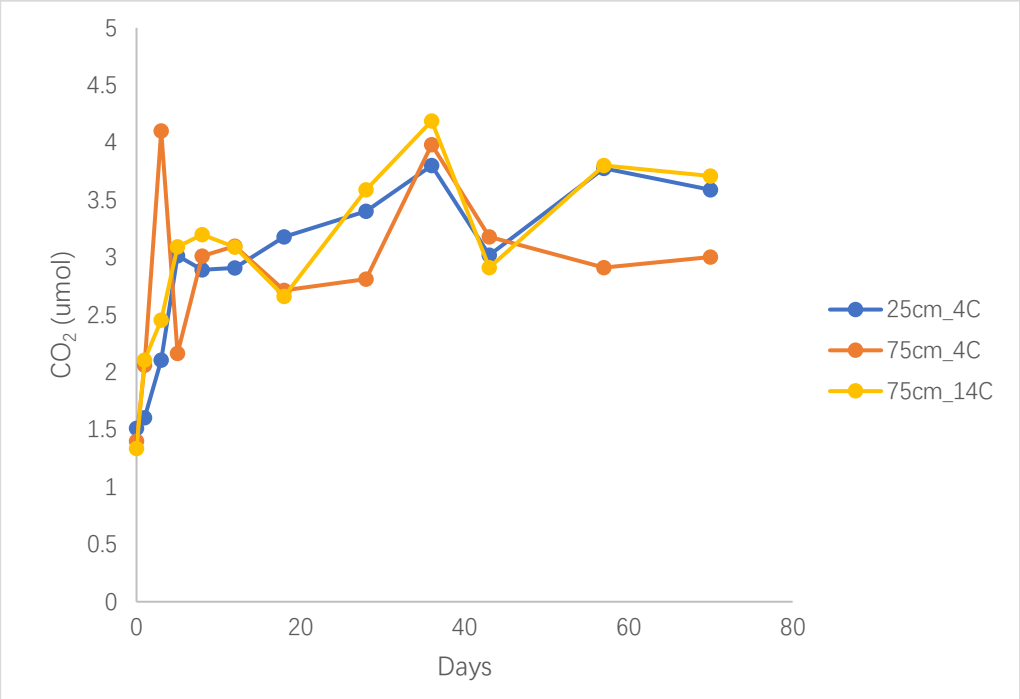



Figure A5: $CO_2$ production over time during pre-incubation of porewater collected from different depths (25 cm and 75 cm) at different incubation temperatures (4 ºC and 14 ºC). At day 50, acetate was added to all treatments.

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